#16

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Applications of:	)	
PRIEELS, J.P. et al.	)	
	)	
Serial No. 08/442,288	)	Group Art Unit: 1813
Filed: May 16, 1995	)	Examiner: L.F. Smith
For: VACCINE COMPOSITIONS	)	
CONTAINING ADJUVANTS	)	
	)	

EXHIBIT BOOK FOR APPELLANTS' BRIEF ON APPEAL

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EXHIBIT 1

APPENDIX

CLAIMS--S.N. 08/356,372

1. A vaccine composition comprising:

(a) an antigen; and antigenic composition and combinations thereof;

(b) QS21 and

(c) 3-De-O-acylated monophosphoryl lipid A (3D-MPL).

2. A vaccine as claimed in claim 1 wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.

3. A vaccine composition as claimed in claim 1 capable of invoking a cytolytic T cell response in a mammal to the antigen or antigenic composition.

4. A vaccine composition as claimed in claim 1 capable of stimulating interferon γ production.

5. A vaccine composition as claimed in claim 2 wherein the ratio of QS21:3D-MPL is form 1:1 to 1:2.5.

6.[1] A vaccine composition as claimed in claim 1 comprising an antigen or antigenic composition derived from the group consisting of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Herpes Simplex Virus type 1, Herpes simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, *Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium* and *Toxoplasma.*

7. A vaccine as claimed in claim 1 wherein the antigen is a tumor antigen.

10. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition according to claim 1.

11. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition according to claim 1.

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[1] By an amendment filed with the brief, Appellants have canceled claim 6.

12. A process for making a vaccine composition according to claim 1 comprising admixing QS21 and 3D-MPL with an antigen, antigenic composition or combination thereof.

13. A vaccine composition as claimed in claim 1 comprising an antigen or antigenic composition derived from the group consisting of Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or #, Respiratory Syncytial virus, human papilloma virus, Influenza virus, *Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium* and *Toxoplasma*.

14. A pharmaceutical composition useful for adjuvanting an immune response comprising an adjuvanting effective combination of QS21 and 3-De-O-acylated monophosphoryl lipid A (3D-MPL).

15. The composition as claimed in claim 14 capable of invoking a cytolytic T cell response in a mammal to an antigen or antigenic composition.

16. The composition as claimed in claim 14 capable of stimulating interferon γ production.

17. The method for stimulating a cytotoxic T cell response in an animal comprising introducing into said animal a cytotoxic T cell response stimulating amount of the composition of claim 1.

18. A method for stimulating γ-interferon response in an animal comprising introducing into said animal a γ-interferon response stimulating amount of the composition of claim 1.

EXHIBIT 2

APPENDIX

CLAIMS--S.N. 08/442,288

1. A vaccine composition comprising:

(a) an antigen; and antigenic compositions and combinations thereof;

(b) QS21 and

(c) 3-De-O-acylated monophosphoryl lipid A (3D-MPL).

2. A vaccine as claimed in claim 1 wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.

3. A vaccine composition as claimed in claim 1 capable of invoking a cytolytic T cell response in a mammal to the antigen or antigenic composition.

4. A vaccine composition as claimed in claim 1 capable of stimulating interferon γ production.

5. A vaccine composition as claimed in claim 2 wherein the ratio of QS21:3D-MPL is from 1:1 to 1:2.5.

6.[1] A vaccine composition as claimed in claim 1 comprising an antigen or antigenic composition derived from the group consisting of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, *Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium* and *Toxoplasma.*

7. A vaccine as claimed in claim 1 wherein the antigen is a tumor antigen.

10. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition according to claim 1.

11. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition according to claim 1.

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[1] By an amendment filed with this brief, Appellants have canceled claim 6.

12. A process for making a vaccine composition according to claim 1 comprising admixing QS21 and 3D-MPL with an antigen, antigenic composition or combination thereof.

13. A vaccine composition as claimed in claim 1 comprising an antigen or antigenic composition derived from the group consisting of Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C, or E, Respiratory Syncytial virus, human papilloma virus, Influenca virus, *Salmonella, neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium* and *Toxoplasma.*

14. A pharmaceutical composition useful for adjuvanting an immune response comprising an adjuvanting effective combination of QS21 and 3-De-O-acylated monophosphoryl lipid A (3D-MPL).

15. The composition as claimed in claim 14 capable of invoking a cytolytic T cell response in a mammal to an antigen or antigenic composition.

16. The composition as claimed in claim 14 capable of stimulating interferon γ production.

17. A method for stimulating a cytotoxic T cell response in an animal comprising introducing into said animal a cytotoxic T cell response stimulating amount of the composition of claim 1.

18. A method for stimulating a γ-interferon response in an animal comprising introducing into said animal a γ-interferon response stimulating amount of the composition of claim 1.

EXHIBIT 3

PATENT
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Atty. Docket 04012.0188

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Applications of: PRIEELS, J.P. et al.	)	
Serial No. 08/442,288 Filed: May 16, 1995 For: VACCINE COMPOSITIONS CONTAINING ADJUVANTS	)	Group Art Unit: 1813 Examiner: L.F. Smith

AMENDMENT AFTER FINAL

Commissioner of Patents and Trademarks
Washington, D.C. 20231

IN THE CLAIMS:

Please cancel claim 6.

REMARKS

Appellants are canceling claim 6 to remove an issue for appeal. Appellants filed a notice of appeal on November 20, 1996 and are filing an appeal brief with this Amendment. In the final Office Action of May 20, 1996, the Examiner had rejected claim 6 under 35 U.S.C. § 112, ¶ 1. No other pending claims are rejected under § 112. By canceling claim 6, Appellants are removing the § 112 issue from this appeal. Entry of this amendment in accordance with MPEP § 1207 is requested.

If there is any fee due for this amendment, including any extension of time, please charge any such fee to Deposit Account No. 06-916.

Date: May 19, 1997

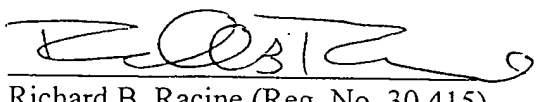

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EXHIBIT 4

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(54) Title: VACCINE COMPOSITION CONTAINING ADJUVANTS

(57) Abstract

The present invention provides vaccine compositions comprising 3 De-O-acylated monophosphoryl lipid A and QS21. The vaccines compositions are potent inducers of CTL and γ IFN responses.

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VACCINE COMPOSITION CONTAINING ADJUVANTS

The present invention relates to novel vaccine formulations, to methods of their production and to their use in medicine. In particular, the present invention relates to vaccines containing QS21, an Hplc purified non-toxic fraction derived from the bark of Quillaja Saponaria Molina, and 3 De-O-acylated monophosphoryl lipid A (3 D-MPL).

3 De-O-acylated monophosphoryl lipid A is known from GB2220 211 (Ribi). Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem Montana.

QS21 is a Hplc purified non toxic fraction of a saponin from the bark of the South American tree Quillaja saponaria molina and its method of its production is disclosed (as QA21) in US patent No. 5,057,540.

The present invention is based on the surprising discovery that formulations containing combinations of QS21 and 3 D-MPL synergistically enhance immune responses to a given antigen.

For example a vaccine formulation of the malarial antigen, RTS, S in combination with 3D-MPL and QS21 results in a powerful synergistic induction of CS protein specific cytotoxic T lymphocyte (CTL) response in the spleen.

RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of P.falciparum linked via four amino acids of the $preS_2$ portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus. It's full structure is disclosed in co-pending International Patent Application No. PCT/EP92/02591, published under Number WO 93/10152 claiming priority from UK patent application No.9124390.7. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S.

The observation that is possible to induce strong cytolytic T lymphocyte responses is significant as these responses, in certain animal models have been shown to induce protection against disease.

The present inventors have shown that the combination of the two adjuvants QS21 and 3D-MPL with the recombinant particulate antigen RTS.S results in a powerful induction of CS protein specific CTL in the spleen. QS21 also enhances induction of CTL on its own, while 3D-MPL does not. The combination can be said to act in a synergistic way, because it has an effect that is larger than the sum of the separate effects of each adjuvant. The synergy between these two adjuvants for CTL induction is a surprising observation which has important implications for the use of recombinant molecules as vaccines for induction of CTL mediated immunity.

Induction of CTL is easily seen when the target antigen is synthesised intracellularly (e.g. in infections by viruses, intracellular bacteria, or in tumours), because peptides generated by proteolytic breakdown of the antigen can enter the appropriate processing pathway, leading to presentation in association with class I molecules on the cell membrane. However, in general, pre-formed soluble antigen does not reach this processing and presentation pathway, and does not elicit class I restricted CTL. Therefore conventional non-living vaccines, while eliciting antibody and T helper responses, do not generally induce CTL mediated immunity. The combination of the two adjuvants QS21 and 3D-MPL can overcome this serious limitation of vaccines based or recombinant proteins, and induce a wider spectrum of immune responses.

CTL specific for CS protein have been shown to protect from malaria in mouse model systems (Romero et al. Nature 341:323 (1989)). In human trials where volunteers were immunised using irradiated sporozoites of P. falciparum, and shown to be protected against subsequent malaria challenge, induction of CTL specific for CS epitopes was demonstrated (Malik et al. Proc. Natl. Acad. Sci. USA 88:3300 (1991)).

The ability to induce CTL specific for an antigen administered as a recombinant molecules is relevant to malaria vaccine development, since

the use of irradiated sporozoites would be impractical, on the grounds of production and the nature of the immune response.

In addition to malaria vaccines, the ability to induce CTL responses would benefit vaccines against herpes simplex virus, cytomegalovirus, human Immunodeficiency virus, and generally all cases where the pathogen has an intracellular life stage.

Likewise, CTL specific for known tumour antigens could be induced by a combination of a recombinant tumour antigen and the two adjuvants. This would allow the development of anti cancer vaccines.

In certain systems, the combination of 3D-MPL and QS21 have been able to synergistically enhance interferon γ production. The present inventors have demonstrated the synergistic potential of 3D-MPL and QS21 by utilising a herpes simplex antigen known as gD_2t. gD_2t is a soluble truncated glycoprotein D from HSV-2 and is produced in CHO cells according to the methodology Berman et al. Science 222 524-527.

IFN-γ secretion is associated with protective responses against intracellular pathogens, including parasites, bacteria and viruses. Activation of macrophages by IFN-γ enhances intracellular killing of microbes and increases expression of Fc receptors. Direct cytotoxicity may also occur, especially in synergism with lymphotoxin (another product of TH1 cells). IFN-γ is also both an inducer and a product of NK cells, which are major innate effectors of protection. TH1 type responses, either through IFN-γ or other mechanisms, provide preferential help for IgG2a immunoglobulin isotypes.

Glycoprotein D is located on the viral envelope, and is also found in the cytoplasm of infected cells (Eisenberg R.J. et al J. of Virol. 1980 35 428-435). It comprises 393 amino acids including a signal peptide and has a molecular weight of approximately 60kD. Of all the HSV envelope glycoproteins this is probably the best characterized (Cohen et al. J. Virology 60 157-166). In vivo it is known to play a central role in viral attachment to cell membranes. Moreover, glycoprotein D has been shown to be able to elict neutralizing antibodies in vivo (Eing et al. J. Med Virology 127: 59-65). However, latent HSV2 virus can still be reactivated

and induce recurrence of the disease despite the presence of high neutralizing antibodies titre in the patients sera. It is therefore apparent that the ability to induce neutralizing antibody alone is insufficient to adequately control the disease.

In order to prevent recurrence of the disease, any vaccine will need to stimulate not only neutralizing antibody, but also cellular immunity mediated through T-cells, particularly cytotoxic T-cells.

In this instance the gD_2t is HSV2 glycoprotein D of 308 amino acids which comprises amino acids 1 though 306 of the naturally occurring glycoprotein with the addition of Asparagine and Glutamine at the C terminal end of the truncated protein. This form of the protein includes the signal peptide which is cleaved to yield a mature 283 amino acid protein. The production of such a protein in Chinese Hamster ovary cells has been described in Genentech's European patent EP-B-139 417.

The mature truncated glycoprotein D (rgD2t) or equivalent proteins secreted from mammalian cells, is preferably used in the vaccine formulations of the present invention.

The formulations of the present invention are very effective in inducing protective immunity in a genital herpes model in guinea pigs. Even with very low doses of antigen (e.g. as low as 5 µg rgD2t) the formulations protect guinea pigs against primary infection and also stimulate specific neutralising antibody responses. The inventors, utilising formulation of the present invention, have also demonstrated Effector cell mediated responses of the TH1 type in mice.

Accordingly, the present invention provides a vaccine or pharmaceutical formulation comprising an antigen in conjunction with 3 Deacylated monophosphoryl lipid A and QS21. Such a formulation is suitable for a broad range of monovalent or polyvalent vaccines.

Preferably the vaccine formulations will contain an antigen or antigenic composition capable of eliciting an immune response against a human or animal pathogen, which antigen or antigenic composition is derived from HIV-1, (such as gp120 or gp160), any of Feline Immunodeficiency virus,

human or animal herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Varicella Zoster Virus (such as gpI, II or III), or from a hepatitis virus such as hepatitis B virus for example Hepatitis B Surface antigen or a derivative thereof, hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as Respiratory Syncytial virus, human papilloma virus or Influenza virus, or derived from bacterial pathogens such as Salmonella, Neisseria, Borrelia (for example OspA or OspB or derivatives thereof), or Chlamydia, or Bordetella for example P.69, PT and FHA, or derived from parasites such as plasmodium or Toxoplasma.

The formulations may also contain an anti-tumour antigen and be useful for immunotherapeutically treating cancers.

The formulation may also be useful for utilising with herpetic light particles such as described in International Patent Application No. PCT/GB92/00824 and, International Patent Application No. PCT/GB92/00179.

Derivatives of Hepatitis B Surface antigen are well known in the art and include, inter alia, those PreS$_1$, PreS$_2$ S antigens set forth described in European Patent applications EP-A-414 374; EP-A-0304 578, and EP 198-474.

In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine.

The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1 : 5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5:1 to 1:1 3D MPL: QS21. Typically for human administration QS21 and 3D MPL will be present in a vaccine in the range 1 µg - 100 µg, preferably 10 µg - 50 µg per dose. Often the vaccine will not require any specific carrier and be formulated in an aqueous or other pharmaceutically acceptable buffer. In some cases it may be advantageous that the vaccines of the present invention will further contain alum or be presented in an oil in water emulsion, or other suitable

vehicle, such as for example, liposomes, microspheres or encapsulated antigen particles.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 2-100 μg, most preferably 4-40 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

The formulations of the present invention maybe used for both prophylatic and therapeutic purposes.

Accordingly in one aspect, the invention provides a method of treatment comprising administering an effective amount of a vaccine of the present invention to a patient.

Examples

1.0 Synergy between 3D MPL and QS21 for induction of Interferon γ secretion.

In order to test the ability of 3D MPL and QS21 based adjuvant formulations of rgD2t, to induce effector cell mediated immune responses, groups of Balb/c mice were vaccinated, and their draining lymph node cells tested for IFN-γ secretion as described below.

1.1 rgD2t formulations

This experiment compared three adjuvant formulations:

i) rgD2t in 3D-MPL
ii) rgD2t in QS21
iii) rgD2t in 3D-MPL/QS21

These formulations were made up as follows. rgD2t was produced in CHO cells and corresponds to the mature 1-283 amino acids of HSV-2 gD and is produced according to the methodology of Berman (supra) and EP 0139417.

* rgD2t / 3D-MPL

5 µg of rgD2t/dose are incubated 1h, under agitation,at room temperature, then mixed with a 3D-MPL suspension (25 µg/dose). The volume is adjusted to 70 µl/dose using a sodium chloride solution (5M, pH 6.5 ± 0.5) and water for injection to obtain a final concentration of 0.15M sodium chloride. pH is kept at 6.5 ± 0.5.

* rgD2t/QS21

5 µg rgD2t/dose are incubated 1h at room temperature under agitation The volume is adjusted using sodium chloride solution (5M, pH 6.5 ± 0.5) and water for injection to 70µl. QS21 (10 µg/dose) is then added. pH is kept at 6.5 ± 0.5.and sodium chloride final concentration at 0.15M.

* rgD2t/3D-MPL / QS21.

5 µg rgD2t/dose are incubated 1h at room temperature under agitation. 3D-MPL (25 µg/dose) is added as an aqueous suspension. The final volume of 70µl is completed by addition of an aqueous solution of QS21 (10 µg/dose) and the pH kept at 6.5 ± 0.5 and the sodium chloride concentration at 0.15M.

1.2 IMMUNISATION

Mice were injected into the hind footpads with 35 µL/footpad of formulation. Thus each mouse received 70 µL. Immunisation were on days 0, and 14. Animals were sacrificed on day 21.

1.3 INTERFERON γ ASSAYS

Popliteal lymph node cells from immunised mice were stimulated in vitro using rgD2t at 10, 1, 0.1, 0 µg/ml. Triplicate cultures (200 µl volumes) were set up in round bottom 96-well microtiter plates, using 2 x 10^5 responder cells and 2 x 10^5 irradiated (3000 rad) syngeneic naive spleen cells. Culture medium was RPMI 1640 with 10% foetal calf serum. Aliquots of 100 µl of culture medium from each replicate were harvested and pooled for IFN-γ determinations. Cultures were assayed at 72 hours. For all assays, a control group using ConA (Boehringer Mannheim) at 5 µg/mL was included. This was always positive.

Secretion of IFN-γ was determined using a commercial ELISA assay manufactured by Holland Biotechnology (distributed by Gibco). Assays were carried out on 100 µl of pooled supernatant from triplicate wells.

Secretion of IFN-γ above the assay background of 50 pg/µl was observed in all three formulation groups (see Table). In addition, a synergistic effect between QS21 and 3D-MPL was observed. While each adjuvant on its own induced cells capable of secreting IFN-γ in response to rgD2t, their combination induced more than twice the sum of individual responses.

1.4 Results

Synergy between QS21 and 3D-MPL for induction of IFN-γ secretion.

Immunization:		QS21/3D-MPL rgD2t	QS21 rgD2t	3D-MPL rgD2t
In vitro	10.0	1351	1105	515
stimulation	1.0	914	116	192
(μg/mL gD2t):	0.1	335	<50	143
	0.0	101	<50	139

IFN-γ is expressed in pg/mL.

The table clearly shows that the combined vaccine induces IFN-γ-secretion in a synergistic manner.

2.0 Synergy Between 3D MPL and QS21 for the induction of CTLs

In order to test the ability of RTS,S particles in 3D MPL and QS21 based adjuvant formulations to induce CTLs, groups of B10.BR mice were immunised and their spleen cells stimulated in vitro and tested in cytotoxicity assays on L cells expressing the CS protein.

2.1 Formulation of RTS,S particles.

RTS,S particles were formulated in three different compositions:

1. RTS,S particles ((10μg) with QS21 (10μg) and 3D-MPL (25μg);
2. RTS,S particles ((10μg) with QS21 (10μg);
3. RTS,S particles ((10μg) with 3D-MPL (25μg);

The formulations were made up as follows:

RTS, S/3 D MPL

10 µg of RTS, S particles/dose was incubated at room temperature under agitation then mixed with a 3D MPL aqueous suspension (25µg/dose). The volume is then adjusted to 70 µl/dose using water for injections and a sodium chloride solution (5N, pH 6.5 ± 0.5) to reach a final concentration of 0.15M sodium chloride (pH is kept at 6.5 ± 0.5).

RTS,S /QS21

10µg of RTS, S particles/dose incubated 1h. at room temperature under agitation. The volume is adjusted using water for injection and a sodium chloride solution (5N, pH 6.5 ± 05) and completed to a final volume of 70µ l/ dose with an aqueous solution of QS21 (10µg/dose). pH is kept at 6.5 ± 0.5 and sodium chloride final concentration at 0.15M.

RTS,S / 3 D MPL / QS21

10 µg of RTS,S particles / dose are incubated 1h. at room teperature under agitation then mixed with a 3D MPL (aqueous suspension (25µg/dose) - The volume is then adjusted with water for injection and a sodium chloride solution (5D pH 6.5 ± 0.5). The final volume is completed by addition of an aqueous solution of QS21 (10µg/dose). pH is kept at 6.5 ± 0.5, and sodium chloride final concentration at 0.15 M.

2.2 Immunisation of mice with RTS,S particles

Four to six week old female mice of the strain B10.BR (H-2^k) were purchased from IFFA CREDO (France). Groups of 3 animals were immunised by intra foot-pad injection of 35 µL of antigen formulation into each hind limb. The animals were boosted with a second equal dose of antigen injected two weeks later.

2.3. In vitro stimulation on anti CS CTL

Two weeks after the boost, spleen cells were harvested and stimulated in vitro using syngeneic fibroblasts transfected with the P. falciparum circumsporozoite protein gene (7G8 clone). These CS-transfectant cells

have been described in the paper by Kumar, S. et al. (1988), Nature 334:258-260.

The cultures were established in RPMI 1640 medium supplemented with 10% of heat inactivated foetal calf serum and usual additives, in conditions well known to those of skill in the art.

Responder cells were cultured at a concentration of 10^6 cells/mL in the presence of 10^5 CS-transfectants per mL. To prevent proliferation of CS-transfectant cells, these were irradiated using a dose of 2×10^4 rad. The cultures were fed by replacing 1/2 of culture medium on day 3 and 6, and tested for cytolytic activity on day 7.

2.4. Cytotoxicity assay for anti-CS CTL

Responder cell cultures were harvested, washed, and mixed at ratios varying from 100:1 to 0.3:1 with a constant number of 2000 target cells, in volumes of 200 µL of medium in V-bottom 96-well plates.
Target cells were syngeneic fibroblast cells that had been labelled with ^{51}Cr.

Two different types of target cells were used:

1. L cells
2. CS transfected L cells

These are described in: Kumar, S. et al. (1988), Nature 334:258-260.

The assay was incubated for 6 hours at 37°C, then the amount of radioactivity released into the supematant by lysis of target cells was determined. Cytolytic activity is expressed as % specific lysis:

Results:

Target cells:	Effector: target ratio	% Specific lysis by formulation: 1. RTS,S/ QS21/ 3D-MPL	2. RTS,S/ QS21/	3. RTS,S/ 3D-MPL
CS transfected L cells	100	58	17	1
	30	53	10	0
	10	47	5	1
	3	27	1	0
	1	11	0	0
	0.3	2	-2	-1
L cell	100	3	-2	5
	30	-2	1	4
	10	0	-1	2
	3	0	3	4
	1	-1	4	2
	0.3	3	1	2

Immunisation of B10.BR mice with RTS,S adjuvanted with QS21 and 3D-MPL (formulation #1) induced in the spleen high levels of CTL specific for the circumsporozoite component of RTS,S. Immunisation with RTS,S particles adjuvanted with QS21 (formulation #2) also induced CTL in the spleen, but only at about 1/30th of the levels given by formulation #1. RTS,S with 3D-MPL (formulation #3) did not induce CTL.

Since the target cells used in this assay do not express MHC class II molecules, the effector cells can be assumed to be $CD8^+$, class I restricted CTL.

3. Other formulation

Hepatitis B Surface Antigen, Alum 3D-MPL and QS21.

The preparation of Hepatitis B Surface antigen (HBsAg) is well documented. See for example Harford et al Develop. Biol. Standard 54 p125 (1983), Gregg et al Biotechnology 5 p479 (1987) EP-A-O 226 846 and EP-A-299 108 and references therein.

3D-MPL was obtained from Ribi Immunochem, QS21 was obtained from Cambridge Biotech, and Aluminium hydroxide was obtained from Superfos (Alhydrogel).

A number of different formulations were made up for studies of cell mediated immunity in mice and for studies in Rhesus monkeys.

3.1 Formulation 1 was made up in phosphate buffer (pH 6.8) to comprise the following per 60 µl dose.

20 µg	HBsAg
30 µg	$Al(OH)_3$
30 µg	3D - MPL
10 µg	QS 21
10 mM	PO_4^{3-}
0.15 M	NaCl

The formulation was made up in the following manner. 20µg HBsAg/dose was incubated with $Al(OH)_3$ for one hour at room temperature with gentle shaking. 3D-MPL was added as an aqueous suspension, and the formulation completed by the addition of QS21, phosphate buffer and sodium chloride and incubated for one hour at room temperature. The final formulation had a pH of between 6.5 and 7.0 and used for foot pad studies in mice.

3.2 Formulation 2 was made up in a phosphate buffer (pH6.8) to comprise the following per 200 µl dose.

1 µg	HBsAg
100 µg	$Al(OH)_3$
50 µg	3D-MPL
20 µg	QS 21
10 mM	PO_4^{3-}
0.15 M	NaCl

The formulation was made up in the following manner. HBsAg and $Al(OH_3)$ were incubated together for one hour at room temperature with

gentle shaking. The formulation was completed by the addition of $Al(OH)_3$, 3D-MPL as an aqueous suspension and QS21, with phosphate buffer and sodium chloride solution and incubated again for thirty minutes. The pH of the formulation was kept between 6.5 and 7.0 and used for Humoral immunity studies in mice.

3.3 Formulation 3 was made up in a similar manner, in a phosphate buffer (pH6.5 - 7.0) to contain the following per 1 ml dose :

10 µg	HBsAg
500 µg	$Al(OH)_3$
50 µg	3D-MPL
10 µg	QS 21

The formulation was used for monkey studies.

4. Conclusions

The combination of the two adjuvants QS21 and 3D-MPL with the recombinant particulate antigen RTS,S resulted in a powerful induction of CS protein specific CTL in the spleen. QS21 enhances induction of CTL on its own, while 3D-MPL does not. The combination can be said to act in a synergistic way, because it has an effect that is larger than the sum of the separate effects of each adjuvant. The synergy between these two adjuvants for CTL induction is a surprising observation which supports our observation of synergy between QS21 and 3D-MPL for induction of T cells capable of secreting IFN-γ in response to stimulation with the soluble recombinant protein gD2t. This finding has important implications for the use of recombinant molecules as vaccines for induction of CTL mediated immunity, since the combination of the two adjuvants QS21 and 3D-MPL can overcome this serious limitation of vaccines based on recombinant proteins, and induce a wider spectrum of immune responses than hitherto.

The mouse cell mediated immunogenicity data show that QS21 based formulations of rgD2t induce a significant synergistic TH1 type T cell response (IFN-γ secretion).

Such TH1 type T cells have been shown to be involved in induction of delayed type hypersensitivity responses in mice. Our own data in prophylaxis of HSV disease show that concomitant induction of neutralizing antibody titers and antigen specific DTH responses affords the best protection against herpes simplex disease.

Put together, these data suggested to us that QS21 formulations of rgD2t may be effective in inducing a protective response against HSV disease. The data presented show an unexpected synergistic effect between 3D Monophosphoryl lipid A and QS21, in inducing IFN-γ secreting antigen specific T cells. Such a synergy may translate in improved ability to induce a protective response against HSV disease, and indeed these formulations are effective in protecting against disease in guinea pigs.

Claims

1. A vaccine composition comprising an antigen and/or antigenic composition, QS21 and 3 De-O-acylated monophosphoryl lipid A (3D-MPL).

2. A vaccine as claimed in claim 1 wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.

3. A vaccine composition as claimed in claim 1 or 2 capable of invoking a cytolytic T cell response in a mammal to the antigen or antigenic composition.

4. A vaccine composition as claimed in any of claims 1 to 3 capable of stimulating interferon γ production.

5. A vaccine composition as claimed in any of claims 1 to 4 wherein the ratio of QS21:3D-MPL is from 1:1 to 1:2.5.

6. A vaccine composition as claimed herein comprising an antigen or antigenic composition derived from any of Human Immunodeficiency Virus, Feline Immunodeficiency Virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A,B,C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma.

7. A vaccine as claimed in any of claim 1 to 5 wherein the antigen is a tumour antigen.

8. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the prophylatic treatment of viral, bacterial, or parasitic infections.

9. Use of composition as defined in any of claims 1 to 5 for the manufacture of a vaccine for the immunotherapeutic treatment of viral, bacterial, parasitic infections or cancer.

10. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.

11. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.

12. A process for making a vaccine composition according to claims 1 to 5 comprising admixing QS21 and 3D-MPL with an antigen or antigenic composition.

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DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I(we) hereby declare that: my(our) residence, post office address and citizenship are as stated below next to my name,

I(we) believe I(we) am(are) the original, first and sole inventor(s) (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

VACCINE COMPOSITION CONTAINING ADJUVANTS
the specification of which
(check one)

[ ] is attached hereto.

[X]was filed on 15 June 1993 as Serial No PCT/EP93/01524
and was amended on (if applicable).

I(we) hereby state that I(we) have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I(we) acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I(we) hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

			Priority Claimed	
Number	Country	Filing Date	Yes	No
9213559.9	Great Britain	25 June 1992	[X]	[ ]
9226283.1	Great Britain	17 December 1992	[X]	[ ]
9304056.6	Great Britain	1 March 1993	[X]	[ ]

I(we) hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section

112. I(we) acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Serial No	Filing Date	Status

I(we) hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Stephen Venetianer	Registration No. 25,659
Stuart R. Suter	Registration No. 26,590
Janice E. Williams	Registration No. 27,142
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I(we) hereby declare that all statements made herein of my(our) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's Signature _____

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Fourth Inventor's signature Pietro Pala

Date 7/12/94

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Citizenship: Italian

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EXHIBIT 5

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/356,372	02/17/95	PRIEELS	

EXAMINER
SMITH, L.

ART UNIT	PAPER NUMBER
1813	12

DATE MAILED: 05/20/96

HERBERT H JERVIS
SMITHKLINE BEECHAM CORPORATION
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This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☐ This application has been examined ☒ Responsive to communication filed on 1/29/96 ☒ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), 0 days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice of Draftsman's Patent Drawing Review, PTO-948.
3. ☒ Notice of Art Cited by Applicant, PTO-1449. 1 page
4. ☐ Notice of Informal Patent Application, PTO-152.
5. ☐ Information on How to Effect Drawing Changes, PTO-1474.
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims 1-7, 10-18 are pending in the application.
   Of the above, claims _____ are withdrawn from consideration.
2. ☒ Claims 8 and 9 have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☒ Claims 1-7, 10-18 are rejected.
5. ☐ Claims _____ are objected to.
6. ☐ Claims _____ are subject to restriction or election requirement.
7. ☐ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.
8. ☐ Formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on _____. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable; ☐ not acceptable (see explanation or Notice of Draftsman's Patent Drawing Review, PTO-948).
10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on _____, has (have) been ☐ approved by the examiner; ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed _____, has been ☐ approved; ☐ disapproved (see explanation).
12. ☒ Acknowledgement is made of the claim for priority under 35 U.S.C. 119. The certified copy has ☒ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____.
13. ☐ Since this application apppears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
14. ☐ Other

EXAMINER'S ACTION

PTOL-326 (Rev. 2/93)

15. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

16. The examiner acknowledges the cancellation of claims 8 and 9 and the addition of claims 13-18.

17. Claims pending are claims 1-7, 10-18.

18. The objection to the specification because of informalities is withdrawn in view of applicant's amendments.

19. The rejection of claims 1-12 under 35 U.S.C. §112 second paragraph as failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is withdrawn in view of applicant's amendments to the claims and cancellation of claims 8 and 9.

20. The examiner acknowledges the substitute specification which is in compliance with 37 CFR 1.52(b) and (c).

21. The examiner acknowledges the preliminary amendment in which claims 1, 3-12 were amended to conform to U.S. practice. The office action mailed 7/24/95 was in fact directed to those claims as amended in the preliminary amendment as all depending from claim 1.

Applicant's arguments filed 1/29/96 have been fully considered but they are not deemed to be persuasive.

22. The provisional double patenting rejection of claims 1-7, 10-12 and newly presented claims 13-18 under 35 U.S.C. §101 is maintained for reasons set forth in paper no. 4,

paragraph 18 of the previous office action. The examiner notes applicant's statement concerning cancelling claims upon notification of allowable subject matter.

23. The objection to the specification and rejection of claim 6 under 35 U.S.C. §112 first paragraph as the disclosure is enabling only for claims limited to an antigenic composition of glycoprotein D and CS protein from malaria in combination with the adjuvants 3D-MPL and QS-21, a method of making the antigenic composition and a method of stimulating cytolytic T cells and gamma interferon production is maintained essentially for reasons set forth in paper no. 4, paragraph 19 of the previous office action.

The rejection was on the grounds that the claims are broadly drawn to a vaccine composition comprising antigen derived from all viral, bacterial or parasitic infections as well as to human immunodeficiency virus and feline leukemia virus. The specification lacks enablement for vaccine compositions which would be effective against all of the claimed species of pathogenic infections, particularly against HIV, prophylactically or therapeutically. The specification provides no probative evidence to support a vaccine which would protect humans against AIDS. The obstacles to vaccine development and therapeutic approaches with regard to retroviruses associated with AIDS in humans are well documented in the literature. These obstacles include: 1) the extensive genomic diversity associated with the HIV retrovirus, particularly with respect to the gene encoding the envelope protein, 2) the fact that the modes of viral transmission include virus-infected mononuclear cells, which pass the infecting virus to other cells in a covert form, as well as via free virus transmission, 3) existence of a latent form of the virus, 4) the ability of the retrovirus to "hide" in the central nervous system where blood cells and neutralizing agents carried by the blood cannot reach the retrovirus, due to the blood-brain barrier and 5) the complexity and variation of the elaboration of the disease. The existence of these obstacles establish that the contemporary knowledge in the art would prevent one of ordinary

skill in the art from accepting any vaccine or any immunization treatment or any therapeutic regimen on its face. In order to enable claims to drugs, antigenic compositions and their uses, either in vivo or in vitro data, or a combination of these can be used. However, the data must be such as to convince one of ordinary skill in the art that the proposed claims are sufficiently enabled. See In re Irons, 340 F.2d 924, 144 USPQ 351 (CCPA 1965), Ex parte Krepelka, 231 USPQ 746 (PTO Bd. Pat. App. & Inter. 1986) and Ex parte Chwang, 231 USPQ 751 (PTO Bd. Pat. App. & Inter. 1986). By definition vaccines must not only induce an immune response, but must be immunogenic to the extent that upon subsequent challenge with the live virus, development of the disease is prevented, or better yet infectivity does not occur.

The development of immune responses important in protection against HIV infection has not been established. Generally it is thought that humoral immunity as well as cell mediated immunity are important in recovery from viral infections. The generation of neutralizing antibody responses against HIV have not been well correlation with slowing or preventing HIV infection. Cohen et al have reported that neutralizing antibodies have been unable to neutralize what is known as "primary field isolates" of HIV, which isolates are more closely related to that which would infect the general population (page 980). Similarly the development of cytolytic responses has not been correlated with the slowing of progression to HIV disease. Butini et al, in comparing CTL activity in lymphoid tissue and peripheral blood found HIV-specific CTL activity in a patient with rapidly progressive disease, while in another patient showing no progression of immunodeficiency, no CTL activity (abstract J306). Thus it is not clear what factors or parameters constitute immunity to HIV disease. Additionally, the claims are drawn to vaccine compositions against feline leukemia virus. This virus appears to be a virus specific to the feline animal species while the others appear to cause infections in humans. The specification lacks enablement for the claimed adjuvant compositions and the feline leukemia virus and enablement to show that the claimed adjuvants are effective in the feline animal species. In view of all of the above and in view of the lack of guidance provided by the specification with respect to the enablement of the broad claims, it is determined that the specification is not commensurate in scope with the claimed subject matter.

Applicant urges that with respect to HIV, clinical trials are proceeding but results are unavailable, the rejection appears to be based upon utility, the specification contains sufficient

data to support the broad scope of the claimed subject matter. Applicant additionally urges that a Declaration under 1.132 will be filed providing additional data if needed and cites In re Vaeck, In re Gardner, and Atlas Powder Co. v. E.I. Dupont de Nemours in support.

It is the examiner's position that claim 6 is broadly drawn to several different species of organisms which include the human immunodeficiency virus. The specification lacks enablement for a vaccine composition, particularly against HIV infection. The obstacles to the development of vaccines and treatment therapies against HIV have already been outlined and discussed in the previous office action. Applicant has stated that antigens form opposite ends of the spectrum were chosen and tested. However, it is not apparent from the specification, that one would reasonably expect particulate or soluble antigens from, for example, HIV to function similarly, particularly in view of the lack of correlation between in vitro results and in vivo efficacy associated with HIV. The immune correlates of HIV infection have yet to be determined and the instant specification lacks sufficient guidance and teaching to show that one would reasonably expect particulate and insoluble HIV antigens to function similarly to CS and glycoprotein D antigens, as disclosed in the instant specification. In view of all of the

above, the specification is not commensurate in scope with the claimed subject matter.

24. The rejection of claims 1, 2, 5, 6, 10, 12 and newly presented claims 13 and 14 under 35 U.S.C. §103 as being unpatentable over Long et al, 1984 in view of Kensil et al, U.S. Pat. No. 5,057,540 and further in view of Schneerson et al, 1991 is maintained essentially for reasons set forth in paper no. 4, paragraph 22 of the previous office action. It should be noted that this is a new grounds of rejection with respect to newly presented claims 13 and 14.

The rejection was on the grounds that Long et al describe the protection of mice from lethal challenge with herpes virus, after administration of herpes virus glycoprotein D in adjuvant (abstract and table 1). The administration of glycoprotein D conferred protection against lethal challenge with both homologous and heterologous virus types (page 763, first column). Glycoprotein D generated high levels of neutralizing antibody titres (pages 761-763 and table 1). Long et al differ from the claimed invention in not specifically describing the use of QS-21 or 3D-MPL in the antigenic composition.

Kensil et al teach compositions of saponins and antigens and the effectiveness of saponins (from *Quillaja saponaria* bark) such as QA-21, QA-17 and QA-18 as adjuvants in antigenic compositions (abstract, figures 12-15 and columns 20-23). Saponins are natural products and may be used as immune adjuvants (col. 3, lines 8-46). The effective ratios of adjuvant to antigen suggested are "3.0 or less or preferably 1.0 or less" (col. 7, lines 10-13). The saponins, particularly QA-21 (col. 5, lines 30-35) which appears to be similar or an obvious or analogous variant of the claimed QS-21, may be administered individually or admixed with other substantially pure adjuvants to "achieve the enhancement of the immune response to an antigen" (col. 7, lines 14-20). While Kensil, et al suggest the use of QA-21 in admixture with other adjuvants, Kensil et al do not specifically describe 3-De-O-acylated monophosphoryl lipid A (3D-MPL) as an adjuvant. However, Schneerson et al describe the enhancement of serum antibody responses in mice to polysaccharide antigens in combination with MPL as adjuvant (abstract, page 213,

tables 1-6). Antigen in combination with MPL, which MPL appears to be similar or an obvious or analogous variant of the claimed 3D-MPL, when administered, generated higher specific serum antibody responses. Schneerson et al also describe compositions of MPL and other adjuvants with antigen at ratios of 1:1 (page 213, tables 2-5). It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to include QS-21 and 3D-MPL in an antigenic composition with antigen as suggested by Kensil et al and Schneerson et al. It would have been expected, barring evidence to the contrary, that the addition of QS-21 and 3D-MPL to glycoprotein D subunit vaccine of herpes simplex virus of Long, et al, would be effective in enhancing the neutralizing antibody response to glycoprotein D, resulting in protection against infection with herpes virus types 1 and 2. The use of a particular ratio of QS-21 to 3D-MPL is well within the level of skill in the art, would be a matter of empirical determination and design choice.

Applicant urges that Long et al disclose gD of herpes simplex in combination with Freund's complete adjuvant, only antibody responses are disclosed, no CTL responses are disclosed, there is no suggestion to use an adjuvant other than Freund's and the last sentence of Long states that it remains to be seen if the test vaccine is protective against the establishment of latency of recurrent infection.

It is the examiner's position that the claims do not recite humoral or CTL responses (i.e. claims 1, 2, 5, 6, 10, 12, 13, 14). The claims are drawn to a vaccine composition comprising antigen and the QS21 and 3D-MPL adjuvants. The claims are also not drawn to a method of inhibiting the establishment of latency of recurrent herpes simplex virus infection. Long et la disclose that immunization with adjuvanted glycoprotein D from Herpes Simplex Virus types 1 and 2 can protect against lethal

challenge with Herpes virus. It is very well known in the art that Freund's complete adjuvant is not suitable for human use. Thus the ordinarily skilled artisan would be motivated to use an adjuvant other than Freund's complete adjuvant, if contemplating human use. However, it should be noted that the claims are not limited to the use of the compositions in humans.

Applicant urges that Kensil et al do not suggest the use of QS-21 in conjunction with 3D-MPL. Applicant urges that Kensil et al disclose the use of mixtures of saponin and/or non-saponin adjuvants but does not suggest that there is synergism with the addition of another adjuvant. Applicant additionally adds that Kensil et al only disclosed one working example to support their broadly claimed subject matter.

It should be noted that neither applicant nor the examiner is knowledgeable of the prosecution history of the application which resulted in U.S. Pat. No. 5,057,540 or what additional evidence was presented or what additional data were presented. It is the examiner's position that applicant appears to argue the references individually without clearly addressing the combination of references. Kensil et al suggest the following:

a) the use of QS-21 adjuvant to enhance immune responses

b) mixtures of the saponin adjuvant which enhanced antibody responses two orders of magnitude greater when the antigen was administered without adjuvant (column 4, lines 20-28)

c) the effectiveness of the saponin adjuvants at lower doses (column 5, lines 28-34)

d) the administration of saponin together with non-saponin adjuvants from a variety of other adjuvant sources (column 7, lines 14-40) and

e) a method of administering a composition comprising antigen and a mixture of saponins (columns 20-22).

Applicant urges that the tertiary reference relates to MPL and not 3D-MPL and only in combination with TDM, there is no suggestion of other adjuvants which can be used and the combination of references is improper citing in support, for example, In re Imperato, In re Nomiya, In re Fine and In re Ochiai. It appears that the cited case law is not particularly relevant to the instant invention. Contrary to the facts presented in for example, In re Nomiya or In re Fine, the cited combination of teachings in the instant application expressly suggests a mixture of antigen and adjuvants and a suggestion of an enhanced immune response which would be two orders of magnitude greater than a response absent adjuvants. Additionally, one of ordinary skill in the art realizing that Freund's Complete Adjuvant is not suitable for human use would be motivated to use an adjuvant other than Freund's Complete Adjuvant particularly if contemplating human use. These facts appear to be contrary to those in the cited case law.

It is the examiner's position that again applicant appears to argue the references individually as if each was anticipatory under 35 U.S.C. §102. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon references which make up the state of the art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413, 208 USPQ 871 (CCPA 1981). Moreover, specific statements in the references themselves which would spell out the claimed invention are not necessary to show obviousness since questions of obviousness involves not only what references expressly teach, but what they would collectively suggest to one of ordinary skill in the art. See CTS Corp. v. Electro Materials Corp. of America (DC SNY) 202 USPQ 22; and In re Burckel (CCPA 201 USPQ 67). It should be noted that it was stated that the adjuvant described by Schneerson et al appears to be an obvious or analogous variant of the claimed adjuvant. There is nothing on record to show that the MPL of the prior art reference would not function similarly to the claimed 3D-MPL adjuvant. Indeed, applicant has submitted a reference (Myers, U.S. Pat. No. 4,912,094) which states that all of the uses disclosed in the literature for MPL can be

entertained with respect to d3-MPL (col. 9, lines 62-68). Thus it would appear that the criticality of using 3D-MPL as opposed to MPL has not been established in the instant specification. The combination of teachings of the prior art has suggested the use of antigen in combination with a mixture of adjuvants (saponin and/or non-saponin), and the art suggests that lower doses of saponin may be used to enhance the immune response at least two orders of magnitude higher. Schneerson et al suggest the use of MPL, which appears to be an obvious variant of the claimed 3D-MPL, in combination with TDM to enhance antibody response to antigen. It would follow then, that the combination of QS-21 + MPL + antigen would at least enhance an immune response two orders of magnitude higher, absent evidence to the contrary. It should be noted that the claims are drawn to a vaccine or pharmaceutical composition employing the open ended terminology "comprising". Applicant's use of the open-ended term "comprising" in the claims fails to exclude unrecited steps and leaves the claims open for inclusion of unspecified ingredients, even in major amounts. See In re Horvitz, 168 F 2d 522, 78 U.S.P.Q. 79 (C.C.P.A. 1948) and Ex parte Davis et al., 80 U.S.P.Q. 448 (PTO d. App. 1948).

25. The rejection of claims 1, 3, 4, and newly presented claims 15-18 under 35 U.S.C. §103 as being obvious over Schofield et al or Weiss et al in view of Kensil et al and further in view

of Schneerson et al is maintained essentially for reasons set forth in paper no. 4, paragraph 23 of the previous office action. It should be noted that this is a new grounds of rejection with respect to newly presented claims 15-18.

The rejection was on the grounds that Schofield et al describe the immunization of rats with irradiated *Plasmodium berghei* sporozoites. The immunization with irradiated sporozoites generated humoral immunity as well as cell mediated immunity with the development of gamma interferon producing (γIFN) cytotoxic T cells, indicating the involvement of cytotoxic cells and γIFN in the development of immunity to malaria sporozoites (page 668). Likewise, Weiss et al describe immunization of mice with live sporozoites and the development of T cell-mediated immunity (abstract, page 573 and tables 1-3). Schofield, et al and Weiss et al differ from the claimed invention in not specifically describing antigenic compositions comprising QS-21 and 3D-MPL. However, the teachings of Kensil et al and Schneerson et al have already been described above. Thus it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to include 3D-MPL and QS-21 in an antigenic composition of live or irradiated sporozoites. It would have been expected, barring evidence to the contrary, that the antigenic composition would generate enhanced cytolytic T cell responses and γIFN production which would result in enhanced immunity to malaria sporozoites.

Applicant urges that Weiss states that CD8+ T cells are required for protection, immunization with irradiated sporozoites is impractical, Schofield et al as well as Weiss et al do not offer solutions to the problem of the need for γ-interferon production and T cell mediated immunity and that neither of the references in combination with the secondary or tertiary references teaches or suggests the claimed invention.

It is the examiner's position that Weiss et al establish the importance of T cell mediated immunity to parasitic infections such as malaria. the antigen employed was irradiated

sporozoites. It is stated that humans have been successfully immunized with irradiated sporozoites. It is also stated that the use of attenuated parasites is impractical. However, it should be noted that the claims are not limited to human malarial organisms or the use of the vaccine composition in humans. Schofield et al additionally establish the need for γ-interferon, CD8+ T cells and antibodies in immunity to the antigen, malaria sporozoites. The combination therefore of irradiated malaria sporozoite antigen as disclosed by Weiss or Schofield with QS-21 and MPL as disclosed by Kensil and Schneerson, respectively, would indeed have been obvious as has already been described above. It would have been expected, barring evidence to the contrary, that immune responses would have been generated with the addition of the mixture of adjuvants which would have resulted in at least two orders of magnitude higher than immune responses in the absence of adjuvants.

26. The rejection of claims 1, 7 and 11 under 35 U.S.C. §103 as being unpatentable over Cantrell, U.S. Pat. No. 4,877,611 in view of Kensil et al , U.S. Pat. No. 5,057,540 is maintained essentially for reasons set forth in paper no. 4, paragraph 24 of the previous office action.

The rejection was on the grounds that Cantrell describes vaccines comprising adjuvants, such as MPL, together with tumor antigens. The MPL appears to be similar or an obvious or analogous variant of the claimed 3D-MPL (abstract and cols. 3-6). It is stated that the vaccines are effective for the treatment and prevention of cancerous tumors (col. 2, lines 41-60) and can

be used to provide a protective and lasting tumor immunity (abstract, col. 10-15). It is suggested that other adjuvants can also be employed with the immunogenic compositions (col. 5, lines 34-42). Cantrell differs from the claimed invention in not specifically describing the use of QS-21 in the anti-tumor composition. However, the teachings of Kensil et al, have already been described above. Thus it would have been *prima facie* obvious to one or ordinary skill in the art at the time the invention was made to include QS-21 and MPL in an anti-tumor antigenic composition. It would have been expected, barring evidence to the contrary, that the amount of anti-tumor composition administered would be safe and effective and would enhance tumor immunity which would be protective and long lasting.

Applicant urges that Cantrell does not teach 3D-MPL, the similarity of 3D-MPL to MPL is not relevant and cites In re Ochiai in support, the reference only teaches MPL in combination with a bacterial immunostimulant and there is no suggestion to combine it with a willow bark extract.

It is the examiner's position that as has already been pointed, that applicant appears to argue the references individually, as if each were presented in support of a rejection under 35 U.S.C. §102. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon references which make up the state of the art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413, 208 USPQ 871 (CCPA 1981). Moreover, specific statements in

the references themselves which would spell out the claimed invention are not necessary to show obviousness since questions of obviousness involves not only what references expressly teach, but what they would collectively suggest to one of ordinary skill in the art. See CTS Corp. v. Electro Materials Corp. of America (DC SNY) 202 USPQ 22; and In re Burckel (CCPA 201 USPQ 67). Cantrell describes vaccine compositions comprising adjuvants such as MPL, which appears to be an obvious or analogous variant of the claimed 3D-MPL, together with tumor antigens. It is stated that the vaccines are effective for the treatment and prevention of cancerous tumors. There is nothing on record to show that the MPL adjuvant of Cantrell would not function similarly to the claimed 3D-MPL adjuvant. Additionally, Cantrell suggests that other adjuvants can also be employed with the immunogenic compositions. Thus it would appear that the combination of tumor antigen + MPL as suggested by Cantrell with the QS-21 of Kensil would indeed enhance the immune response and it would have been expected, barring evidence to the contrary, that the immune response would be enhanced at least two orders of magnitude higher than the composition without adjuvant.

27. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. § 1.136(a).

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION

IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 C.F.R. § 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

28. Papers related to this application may be submitted to Group 180 by facsimile transmission. Papers should be faxed to Lynette F. Smith, Art Unit 1813 and should be marked "OFFICIAL" for entry into prosecution history or "DRAFT" for consideration by the examiner without entry. The Art Unit 1813 FAX telephone number is (703)-305-7939. FAX machines will be available to receive transmissions 24 hours a day. In compliance with 1096 OG 30, the filing date accorded to each OFFICIAL fax transmission will be determined by the FAX machine's stamped date found on the last page of the transmission, unless that date is a Saturday, Sunday or Federal Holiday with the District of Columbia, in which case the OFFICIAL date of receipt will be the next business day.

29. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lynette F. Smith whose telephone number is (703) 308-3909.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Should the examiner be unavailable, Supervisory Patent Examiner Christine M. Nucker, may be reached at (703) 308-4028.

Smith/lfs LFS
May 15, 1996

L. F. Smith
LYNETTE F. SMITH
PATENT EXAMINER
GROUP 1800

Form PTO-1449 U.S. Department of Commerce Patent and Trademark Office	ATTY. DOCKET NO. B45036	SERIAL NO. 08/356,372
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICANT Prieels, J.P., et al.	
(Use several sheets if necessary)	FILING DATE February 17, 1995	GROUP 1813

U.S. PATENT DOCUMENTS

Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date If Appropriate
LP	AC	4,912,094	3/27/90	Myers, et al.	C07H	1/00	6/29/88

FOREIGN PATENT DOCUMENTS

		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

EXAMINER	DATE CONSIDERED
L. Smith	5/14/96

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copyof this form with next communication to applicant.

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SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/442,298	05/16/95	PRIEELS	

EXAMINER: SMITH,L

18N1/0520

HERBERT H JERVIS
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KING OF PRUSSIA PA 19406-0939

ART UNIT	PAPER NUMBER
1813	9

DATE MAILED: 05/20/96

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☐ This application has been examined ☒ Responsive to communication filed on 1/29/96 ☒ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), ϕ days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice of Draftsman's Patent Drawing Review, PTO-948.
3. ☒ Notice of Art Cited by Applicant, PTO-1449. 1 page
4. ☐ Notice of Informal Patent Application, PTO-152.
5. ☐ Information on How to Effect Drawing Changes, PTO-1474.
6. ☐ ______

Part II SUMMARY OF ACTION

1. ☒ Claims 1-7, 10-18 are pending in the application.
   Of the above, claims ______ are withdrawn from consideration.
2. ☒ Claims 8 and 9 have been cancelled.
3. ☐ Claims ______ are allowed.
4. ☒ Claims 1-7 10-18 are rejected.
5. ☐ Claims ______ are objected to.
6. ☐ Claims ______ are subject to restriction or election requirement.
7. ☐ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.
8. ☐ Formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on ______. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable; ☐ not acceptable (see explanation or Notice of Draftsman's Patent Drawing Review, PTO-948).
10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on ______, has (have) been ☐ approved by the examiner; ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed ______, has been ☐ approved; ☐ disapproved (see explanation).
12. ☒ Acknowledgement is made of the claim for priority under 35 U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☒ been filed in parent application, serial no. 08/356,372; filed on 2/17/95.
13. ☐ Since this application apppears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
14. ☐ Other

EXAMINER'S ACTION

PTOL-326 (Rev. 2/93)

15. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

16. The examiner acknowledges the cancellation of claims 8 and 9 and the addition of claims 13-18.

17. Claims pending are claims 1-7, 10-18.

18. The objection to the specification because of informalities is withdrawn in view of applicant's amendments.

19. The rejection of claims 1-12 under 35 U.S.C. §112 second paragraph as failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is withdrawn in view of applicant's amendments to the claims and cancellation of claims 8 and 9.

20. The examiner acknowledges the substitute specification which is in compliance with 37 CFR 1.52(b) and (c).

21. The examiner acknowledges the preliminary amendment in which claims 1, 3-12 were amended to conform to U.S. practice. The office action mailed 7/24/95 was in fact directed to those claims as amended in the preliminary amendment as all depending from claim 1.

Applicant's arguments filed 1/29/96 have been fully considered but they are not deemed to be persuasive.

22. The provisional double patenting rejection of claims 1-7, 10-12 and newly presented claims 13-18 under 35 U.S.C. §101 is maintained for reasons set forth in paper no. 4,

paragraph 18 of the previous office action. The examiner notes applicant's statement concerning cancelling claims upon notification of allowable subject matter.

23. The objection to the specification and rejection of claim 6 under 35 U.S.C. §112 first paragraph as the disclosure is enabling only for claims limited to an antigenic composition of glycoprotein D and CS protein from malaria in combination with the adjuvants 3D-MPL and QS-21, a method of making the antigenic composition and a method of stimulating cytolytic T cells and gamma interferon production is maintained essentially for reasons set forth in paper no. 4, paragraph 19 of the previous office action.

The rejection was on the grounds that the claims are broadly drawn to a vaccine composition comprising antigen derived from all viral, bacterial or parasitic infections as well as to human immunodeficiency virus and feline leukemia virus. The specification lacks enablement for vaccine compositions which would be effective against all of the claimed species of pathogenic infections, particularly against HIV, prophylactically or therapeutically. The specification provides no probative evidence to support a vaccine which would protect humans against AIDS. The obstacles to vaccine development and therapeutic approaches with regard to retroviruses associated with AIDS in humans are well documented in the literature. These obstacles include: 1) the extensive genomic diversity associated with the HIV retrovirus, particularly with respect to the gene encoding the envelope protein, 2) the fact that the modes of viral transmission include virus-infected mononuclear cells, which pass the infecting virus to other cells in a covert form, as well as via free virus transmission, 3) existence of a latent form of the virus, 4) the ability of the retrovirus to "hide" in the central nervous system where blood cells and neutralizing agents carried by the blood cannot reach the retrovirus, due to the blood-brain barrier and 5) the complexity and variation of the elaboration of the disease. The existence of these obstacles establish that the contemporary knowledge in the art would prevent one of ordinary

skill in the art from accepting any vaccine or any immunization treatment or any therapeutic regimen on its face. In order to enable claims to drugs, antigenic compositions and their uses, either in vivo or in vitro data, or a combination of these can be used. However, the data must be such as to convince one of ordinary skill in the art that the proposed claims are sufficiently enabled. See In re Irons, 340 F.2d 924, 144 USPQ 351 (CCPA 1965), Ex parte Krepelka, 231 USPQ 746 (PTO Bd. Pat. App. & Inter. 1986) and Ex parte Chwang, 231 USPQ 751 (PTO Bd. Pat. App. & Inter. 1986). By definition vaccines must not only induce an immune response, but must be immunogenic to the extent that upon subsequent challenge with the live virus, development of the disease is prevented, or better yet infectivity does not occur.

The development of immune responses important in protection against HIV infection has not been established. Generally it is thought that humoral immunity as well as cell mediated immunity are important in recovery from viral infections. The generation of neutralizing antibody responses against HIV have not been well correlation with slowing or preventing HIV infection. Cohen et al have reported that neutralizing antibodies have been unable to neutralize what is known as "primary field isolates" of HIV, which isolates are more closely related to that which would infect the general population (page 980). Similarly the development of cytolytic responses has not been correlated with the slowing of progression to HIV disease. Butini et al, in comparing CTL activity in lymphoid tissue and peripheral blood found HIV-specific CTL activity in a patient with rapidly progressive disease, while in another patient showing no progression of immunodeficiency, no CTL activity (abstract J306). Thus it is not clear what factors or parameters constitute immunity to HIV disease. Additionally, the claims are drawn to vaccine compositions against feline leukemia virus. This virus appears to be a virus specific to the feline animal species while the others appear to cause infections in humans. The specification lacks enablement for the claimed adjuvant compositions and the feline leukemia virus and enablement to show that the claimed adjuvants are effective in the feline animal species. In view of all of the above and in view of the lack of guidance provided by the specification with respect to the enablement of the broad claims, it is determined that the specification is not commensurate in scope with the claimed subject matter.

Applicant urges that with respect to HIV, clinical trials are proceeding but results are unavailable, the rejection appears to be based upon utility, the specification contains sufficient

data to support the broad scope of the claimed subject matter. Applicant additionally urges that a Declaration under 1.132 will be filed providing additional data if needed and cites In re Vaeck, In re Gardner, and Atlas Powder Co. v. E.I. Dupont de Nemours in support.

It is the examiner's position that claim 6 is broadly drawn to several different species of organisms which include the human immunodeficiency virus. The specification lacks enablement for a vaccine composition, particularly against HIV infection. The obstacles to the development of vaccines and treatment therapies against HIV have already been outlined and discussed in the previous office action. Applicant has stated that antigens form opposite ends of the spectrum were chosen and tested. However, it is not apparent from the specification, that one would reasonably expect particulate or soluble antigens from, for example, HIV to function similarly, particularly in view of the lack of correlation between in vitro results and in vivo efficacy associated with HIV. The immune correlates of HIV infection have yet to be determined and the instant specification lacks sufficient guidance and teaching to show that one would reasonably expect particulate and insoluble HIV antigens to function similarly to CS and glycoprotein D antigens, as disclosed in the instant specification. In view of all of the

above, the specification is not commensurate in scope with the claimed subject matter.

24. The rejection of claims 1, 2, 5, 6, 10, 12 and newly presented claims 13 and 14 under 35 U.S.C. §103 as being unpatentable over Long et al, 1984 in view of Kensil et al, U.S. Pat. No. 5,057,540 and further in view of Schneerson et al, 1991 is maintained essentially for reasons set forth in paper no. 4, paragraph 22 of the previous office action. It should be noted that this is a new grounds of rejection with respect to newly presented claims 13 and 14.

The rejection was on the grounds that Long et al describe the protection of mice from lethal challenge with herpes virus, after administration of herpes virus glycoprotein D in adjuvant (abstract and table 1). The administration of glycoprotein D conferred protection against lethal challenge with both homologous and heterologous virus types (page 763, first column). Glycoprotein D generated high levels of neutralizing antibody titres (pages 761-763 and table 1). Long et al differ from the claimed invention in not specifically describing the use of QS-21 or 3D-MPL in the antigenic composition.

Kensil et al teach compositions of saponins and antigens and the effectiveness of saponins (from *Quillaja saponaria* bark) such as QA-21, QA-17 and QA-18 as adjuvants in antigenic compositions (abstract, figures 12-15 and columns 20-23). Saponins are natural products and may be used as immune adjuvants (col. 3, lines 8-46). The effective ratios of adjuvant to antigen suggested are "3.0 or less or preferably 1.0 or less" (col. 7, lines 10-13). The saponins, particularly QA-21 (col. 5, lines 30-35) which appears to be similar or an obvious or analogous variant of the claimed QS-21, may be administered individually or admixed with other substantially pure adjuvants to "achieve the enhancement of the immune response to an antigen" (col. 7, lines 14-20). While Kensil, et al suggest the use of QA-21 in admixture with other adjuvants, Kensil et al do not specifically describe 3-De-O-acylated monophosphoryl lipid A (3D-MPL) as an adjuvant. However, Schneerson et al describe the enhancement of serum antibody responses in mice to polysaccharide antigens in combination with MPL as adjuvant (abstract, page 213,

tables 1-6). Antigen in combination with MPL, which MPL appears to be similar or an obvious or analogous variant of the claimed 3D-MPL, when administered, generated higher specific serum antibody responses. Schneerson et al also describe compositions of MPL and other adjuvants with antigen at ratios of 1:1 (page 213, tables 2-5). It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to include QS-21 and 3D-MPL in an antigenic composition with antigen as suggested by Kensil et al and Schneerson et al. It would have been expected, barring evidence to the contrary, that the addition of QS-21 and 3D-MPL to glycoprotein D subunit vaccine of herpes simplex virus of Long, et al, would be effective in enhancing the neutralizing antibody response to glycoprotein D, resulting in protection against infection with herpes virus types 1 and 2. The use of a particular ratio of QS-21 to 3D-MPL is well within the level of skill in the art, would be a matter of empirical determination and design choice.
Applicant urges that Long et al disclose gD of herpes simplex in combination with Freund's complete adjuvant, only antibody responses are disclosed, no CTL responses are disclosed, there is no suggestion to use an adjuvant other than Freund's and the last sentence of Long states that it remains to be seen if the test vaccine is protective against the establishment of latency of recurrent infection.

It is the examiner's position that the claims do not recite humoral or CTL responses (i.e. claims 1, 2, 5, 6, 10, 12, 13, 14). The claims are drawn to a vaccine composition comprising antigen and the QS21 and 3D-MPL adjuvants. The claims are also not drawn to a method of inhibiting the establishment of latency of recurrent herpes simplex virus infection. Long et la disclose that immunization with adjuvanted glycoprotein D from Herpes Simplex Virus types 1 and 2 can protect against lethal

challenge with Herpes virus. It is very well known in the art that Freund's complete adjuvant is not suitable for human use. Thus the ordinarily skilled artisan would be motivated to use an adjuvant other than Freund's complete adjuvant, if contemplating human use. However, it should be noted that the claims are not limited to the use of the compositions in humans.

Applicant urges that Kensil et al do not suggest the use of QS-21 in conjunction with 3D-MPL. Applicant urges that Kensil et al disclose the use of mixtures of saponin and/or non-saponin adjuvants but does not suggest that there is synergism with the addition of another adjuvant. Applicant additionally adds that Kensil et al only disclosed one working example to support their broadly claimed subject matter.

It should be noted that neither applicant nor the examiner is knowledgeable of the prosecution history of the application which resulted in U.S. Pat. No. 5,057,540 or what additional evidence was presented or what additional data were presented. It is the examiner's position that applicant appears to argue the references individually without clearly addressing the combination of references. Kensil et al suggest the following:

a) the use of QS-21 adjuvant to enhance immune responses

b) mixtures of the saponin adjuvant which enhanced antibody responses two orders of magnitude greater when the antigen was administered without adjuvant (column 4, lines 20-28)

c) the effectiveness of the saponin adjuvants at lower doses (column 5, lines 28-34)

d) the administration of saponin together with non-saponin adjuvants from a variety of other adjuvant sources (column 7, lines 14-40) and

e) a method of administering a composition comprising antigen and a mixture of saponins (columns 20-22).

Applicant urges that the tertiary reference relates to MPL and not 3D-MPL and only in combination with TDM, there is no suggestion of other adjuvants which can be used and the combination of references is improper citing in support, for example, In re Imperato, In re Nomiya, In re Fine and In re Ochiai. It appears that the cited case law is not particularly relevant to the instant invention. Contrary to the facts presented in for example, In re Nomiya or In re Fine, the cited combination of teachings in the instant application expressly suggests a mixture of antigen and adjuvants and a suggestion of an enhanced immune response which would be two orders of magnitude greater than a response absent adjuvants. Additionally, one of ordinary skill in the art realizing that Freund's Complete Adjuvant is not suitable for human use would be motivated to use an adjuvant other than Freund's Complete Adjuvant particularly if contemplating human use. These facts appear to be contrary to those in the cited case law.

It is the examiner's position that again applicant appears to argue the references individually as if each was anticipatory under 35 U.S.C. §102. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon references which make up the state of the art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413, 208 USPQ 871 (CCPA 1981). Moreover, specific statements in the references themselves which would spell out the claimed invention are not necessary to show obviousness since questions of obviousness involves not only what references expressly teach, but what they would collectively suggest to one of ordinary skill in the art. See CTS Corp. v. Electro Materials Corp. of America (DC SNY) 202 USPQ 22; and In re Burckel (CCPA 201 USPQ 67). It should be noted that it was stated that the adjuvant described by Schneerson et al appears to be an obvious or analogous variant of the claimed adjuvant. There is nothing on record to show that the MPL of the prior art reference would not function similarly to the claimed 3D-MPL adjuvant. Indeed, applicant has submitted a reference (Myers, U.S. Pat. No. 4,912,094) which states that all of the uses disclosed in the literature for MPL can be

entertained with respect to d3-MPL (col. 9, lines 62-68). Thus it would appear that the criticality of using 3D-MPL as opposed to MPL has not been established in the instant specification. The combination of teachings of the prior art has suggested the use of antigen in combination with a mixture of adjuvants (saponin and/or non-saponin), and the art suggests that lower doses of saponin may be used to enhance the immune response at least two orders of magnitude higher. Schneerson et al suggest the use of MPL, which appears to be an obvious variant of the claimed 3D-MPL, in combination with TDM to enhance antibody response to antigen. It would follow then, that the combination of QS-21 + MPL + antigen would at least enhance an immune response two orders of magnitude higher, absent evidence to the contrary. It should be noted that the claims are drawn to a vaccine or pharmaceutical composition employing the open ended terminology "comprising". Applicant's use of the open-ended term "comprising" in the claims fails to exclude unrecited steps and leaves the claims open for inclusion of unspecified ingredients, even in major amounts. See In re Horvitz, 168 F 2d 522, 78 U.S.P.Q. 79 (C.C.P.A. 1948) and Ex parte Davis et al., 80 U.S.P.Q. 448 (PTO d. App. 1948).

25. The rejection of claims 1, 3, 4, and newly presented claims 15-18 under 35 U.S.C. §103 as being obvious over Schofield et al or Weiss et al in view of Kensil et al and further in view

of Schneerson et al is maintained essentially for reasons set forth in paper no. 4, paragraph 23 of the previous office action. It should be noted that this is a new grounds of rejection with respect to newly presented claims 15-18.

The rejection was on the grounds that Schofield et al describe the immunization of rats with irradiated *Plasmodium berghei* sporozoites. The immunization with irradiated sporozoites generated humoral immunity as well as cell mediated immunity with the development of gamma interferon producing (γIFN) cytotoxic T cells, indicating the involvement of cytotoxic cells and γIFN in the development of immunity to malaria sporozoites (page 668). Likewise, Weiss et al describe immunization of mice with live sporozoites and the development of T cell-mediated immunity (abstract, page 573 and tables 1-3). Schofield, et al and Weiss et al differ from the claimed invention in not specifically describing antigenic compositions comprising QS-21 and 3D-MPL. However, the teachings of Kensil et al and Schneerson et al have already been described above. Thus it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to include 3D-MPL and QS-21 in an antigenic composition of live or irradiated sporozoites. It would have been expected, barring evidence to the contrary, that the antigenic composition would generate enhanced cytolytic T cell responses and γIFN production which would result in enhanced immunity to malaria sporozoites.

Applicant urges that Weiss states that CD8+ T cells are required for protection, immunization with irradiated sporozoites is impractical, Schofield et al as well as Weiss et al do not offer solutions to the problem of the need for γ-interferon production and T cell mediated immunity and that neither of the references in combination with the secondary or tertiary references teaches or suggests the claimed invention.

It is the examiner's position that Weiss et al establish the importance of T cell mediated immunity to parasitic infections such as malaria. the antigen employed was irradiated

sporozoites. It is stated that humans have been successfully immunized with irradiated sporozoites. It is also stated that the use of attenuated parasites is impractical. However, it should be noted that the claims are not limited to human malarial organisms or the use of the vaccine composition in humans. Schofield et al additionally establish the need for γ-interferon, CD8+ T cells and antibodies in immunity to the antigen, malaria sporozoites. The combination therefore of irradiated malaria sporozoite antigen as disclosed by Weiss or Schofield with QS-21 and MPL as disclosed by Kensil and Schneerson, respectively, would indeed have been obvious as has already been described above. It would have been expected, barring evidence to the contrary, that immune responses would have been generated with the addition of the mixture of adjuvants which would have resulted in at least two orders of magnitude higher than immune responses in the absence of adjuvants.

26. The rejection of claims 1, 7 and 11 under 35 U.S.C. §103 as being unpatentable over Cantrell, U.S. Pat. No. 4,877,611 in view of Kensil et al , U.S. Pat. No. 5,057,540 is maintained essentially for reasons set forth in paper no. 4, paragraph 24 of the previous office action.

The rejection was on the grounds that Cantrell describes vaccines comprising adjuvants, such as MPL, together with tumor antigens. The MPL appears to be similar or an obvious or analogous variant of the claimed 3D-MPL (abstract and cols. 3-6). It is stated that the vaccines are effective for the treatment and prevention of cancerous tumors (col. 2, lines 41-60) and can

be used to provide a protective and lasting tumor immunity (abstract, col. 10-15). It is suggested that other adjuvants can also be employed with the immunogenic compositions (col. 5, lines 34-42). Cantrell differs from the claimed invention in not specifically describing the use of QS-21 in the anti-tumor composition. However, the teachings of Kensil et al, have already been described above. Thus it would have been *prima facie* obvious to one or ordinary skill in the art at the time the invention was made to include QS-21 and MPL in an anti-tumor antigenic composition. It would have been expected, barring evidence to the contrary, that the amount of anti-tumor composition administered would be safe and effective and would enhance tumor immunity which would be protective and long lasting.

Applicant urges that Cantrell does not teach 3D-MPL, the similarity of 3D-MPL to MPL is not relevant and cites In re Ochiai in support, the reference only teaches MPL in combination with a bacterial immunostimulant and there is no suggestion to combine it with a willow bark extract.

It is the examiner's position that as has already been pointed, that applicant appears to argue the references individually, as if each were presented in support of a rejection under 35 U.S.C. §102. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon references which make up the state of the art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413, 208 USPQ 871 (CCPA 1981). Moreover, specific statements in

the references themselves which would spell out the claimed invention are not necessary to show obviousness since questions of obviousness involves not only what references expressly teach, but what they would collectively suggest to one of ordinary skill in the art. See CTS Corp. v. Electro Materials Corp. of America (DC SNY) 202 USPQ 22; and In re Burckel (CCPA 201 USPQ 67). Cantrell describes vaccine compositions comprising adjuvants such as MPL, which appears to be an obvious or analogous variant of the claimed 3D-MPL, together with tumor antigens. It is stated that the vaccines are effective for the treatment and prevention of cancerous tumors. There is nothing on record to show that the MPL adjuvant of Cantrell would not function similarly to the claimed 3D-MPL adjuvant. Additionally, Cantrell suggests that other adjuvants can also be employed with the immunogenic compositions. Thus it would appear that the combination of tumor antigen + MPL as suggested by Cantrell with the QS-21 of Kensil would indeed enhance the immune response and it would have been expected, barring evidence to the contrary, that the immune response would be enhanced at least two orders of magnitude higher than the composition without adjuvant.

27. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. § 1.136(a).

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION

IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 C.F.R. § 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

28. Papers related to this application may be submitted to Group 180 by facsimile transmission. Papers should be faxed to Lynette F. Smith, Art Unit 1813 and should be marked "OFFICIAL" for entry into prosecution history or "DRAFT" for consideration by the examiner without entry. The Art Unit 1813 FAX telephone number is (703)-305-7939. FAX machines will be available to receive transmissions 24 hours a day. In compliance with 1096 OG 30, the filing date accorded to each OFFICIAL fax transmission will be determined by the FAX machine's stamped date found on the last page of the transmission, unless that date is a Saturday, Sunday or Federal Holiday with the District of Columbia, in which case the OFFICIAL date of receipt will be the next business day.

29. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lynette F. Smith whose telephone number is (703) 308-3909.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Should the examiner be unavailable, Supervisory Patent Examiner Christine M. Nucker, may be reached at (703) 308-4028.

Smith/lfs
May 15, 1996

L. F. Smith
LYNETTE F. SMITH
PATENT EXAMINER
GROUP 1800

Form PTO-1449 U.S. Department of Commerce Patent and Trademark Office	ATTY. DOCKET NO. B45036C1	SERIAL NO. 08/442,288
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	APPLICANT Prieels, J.P., et al.	
(Use several sheets if necessary)	FILING DATE May 16, 1995	GROUP 1813

U.S. PATENT DOCUMENTS

Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date If Appropriate
LP	AC	4,912,094	3/27/90	Myers, et al.	C07H	1/00	6/29/88

FOREIGN PATENT DOCUMENTS

		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

EXAMINER	DATE CONSIDERED
L. Smith	5/12/96

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copyof this form with next communication to applicant.

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EXHIBIT 6

INFECTION AND IMMUNITY, Feb. 1984, p. 761–764
0019-9567/84/020761-04$02.00/0

Vol. 37, No. 2

Glycoprotein D Protects Mice Against Lethal Challenge with Herpes Simplex Virus Types 1 and 2

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Glycoprotein D is a virion envelope component of herpes simplex virus types 1 and 2. Sets of mice were immunized with purified gD-1 or gD-2 and were challenged with a lethal dose of herpes simple virus, either type 1 or type 2. All or virtually all of the immunized mice survived challenge with either agent, whereas challenge of sham-immunized mice was almost always fatal. Serum samples taken before challenge contained gD-specific antibodies which had 50% neutralization titers ranging from 1:16 to 1:512 against homologous and heterologous virus types. We conclude that either gD-1 or gD-2 is a potential candidate for a subunit vaccine against herpetic infections.

Herpes simplex viruses (HSV) are the causative agents of a number of human diseases, including cold sores, encephalitis, and eye and genital infections (14). The herpes simplex virion envelope and the plasma membrane of HSV-infected cells contain a series of glycoproteins designated gB, gC, gD, and gE (26), all of which appear to be involved in the immune response (15, 27). An additional glycoprotein, gA, is probably a modified form of gB (7). Our laboratory has focused much of its effort on studies of gD. Recently, we described a simple method for purifying gD (9), and we therefore considered the possibility of testing its protective capacity in a mouse model system. A number of studies have shown that gD is a reasonable choice for such a study. gD is a type-common component whose polypeptide and carbohydrate structure appears to be the same in different strains of HSV type 1 (HSV-1) (Cohen and Eisenberg, unpublished data) and is highly conserved between HSV-1 and HSV-2 (8, 18). Purified gD stimulates high titers of complement-independent, type-common virus-neutralizing antibodies in animal systems (4, 5, 9, 19). In addition, gD and other HSV glycoproteins participate in antibody-dependent complement-mediated (1, 16) and antibody-dependent cell-mediated (1, 16, 22) cytotoxic reactions. Passive immunization with monoclonal antibodies directed against gD as well as other HSV glycoproteins is highly effective in protecting mice against challenge by a lethal dose of HSV (10, 16, 22). These studies document the involvement of gD in the immune response to HSV and suggest that gD is an important candidate for a potential subunit vaccine. Live virus, killed virus, and subunit vaccines consisting of a mixture of HSV glycoproteins have been shown in protection studies to be effective (3, 11, 12, 20, 25, 28). More recently (23), purified gC of HSV-1 has been shown to protect mice against a challenge by a lethal dose of HSV-1 but not against HSV-2. The present study evaluates the capacity of purified gD-1 and gD-2 to protect mice against lethal HSV challenge by the homologous and heterologous virus types.

(A portion of this work was presented by T. J. Madara in partial fulfillment of the requirements for an M.S. degree at the University of Pennsylvania, Philadelphia, 1981.)

The intraperitoneal route in mice was chosen for both immunization and lethal virus challenge (see Table 1, footnote *a*, for details). Animals were immunized with affinity-purified gD (9) suspended in complete Freund adjuvant (CFA) because this route-and-adjuvant protocol produced the highest titers of virus-neutralizing antibody. In a preliminary experiment designed to determine the dose of gD needed to stimulate production of neutralizing antibody, groups of mice were given five injections of gD-1 ranging from 0.005 to 2 μg per injection. At a dose of 0.05 μg per injection, two of four mice (50%) responded by producing virus-neutralizing antibody specific for gD (9). On the other hand, 100% of mice receiving doses ranging from 0.5 to 2.0 μg per injection responded by producing gD-specific neutralizing antibody. Based on these data, a regimen of six injections with a total of 6 μg of gD-1 or gD-2 over the course of immunization was chosen for subsequent challenge experiments.

In the first challenge experiment, mice were immunized with gD-1 purified from KB cells infected with strain HF of HSV-1 (9) and were challenged with either the Patton strain of HSV-1 or the 186 strain of HSV-2. In this and the subsequent challenge experiment, serum samples were obtained before virus challenge to be assayed for virus-neutralizing antibody by the 50% plaque reduction method (4, 5, 9) and to be assayed for gD specificity by radioimmune precipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4, 5, 9). Neutralization titers of the immunized mice to be challenged ranged from 1:32 to 1:128 against HSV-1 (the homologous virus) and from 1:16 to 1:128 against HSV-2 (the heterologous virus). All of the immunized mice in this experiment displayed gD-1-specific antibody (data not shown). The sera of mice sham immunized with CFA or with saline showed neither neutralizing antibody nor immunoprecipitating activity with gD-1.

The immunized mice were arranged into two challenge groups, each of which represented the range of neutralization titers against HSV-1 and HSV-2. Table 1 illustrates the capacity of purified native gD-1 (9) to protect mice against lethal challenge with HSV-1 or HSV-2. All of the immunized mice survived the challenge with either HSV-1 or HSV-2. No survivors remained in the mice sham immunized with saline. However, there were some survivors (18 to 33%) among the CFA-immunized mice. These results indicate that gD-1 was effective in protecting mice, including those animals with low neutralization titers (1:16, 1:32), against

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TABLE 1. Protection of mice against lethal challenge by HSV-1 or HSV-2 after immunization with purified gD-1 or gD-2

Immunization group[a]	Challenge virus[b]	Survivors[c]	
		No. total tested	%
Expt 1			
gD-1/CFA	HSV-1 (Patton)	10/10	100
CFA	HSV-1	1/3	33
NaCl	HSV-1	0/9	0
gD-1/CFA	HSV-2 (186)	8/8	100
CFA	HSV-2	2/11	18
Expt 2			
gD-1/CFA	HSV-2 (186)	5/6[d]	83
gD-2/CFA	HSV-2	6/6	100
CFA	HSV-2	0/6	0
NaCl	HSV-2	0/6	0

[a] Survivors are those alive 21 days after challenge.

[b] In both experiments, mice were immunized with a total of 6 μg of purified gD (9). In experiment 1, BALB/c mice were immunized six times at biweekly intervals by intraperitoneal injection of 1.0 μg (dosage and protocol previously established to yield anti-gD responses in 100% of immunized animals) of purified native gD-1 (HF strain) emulsified in 50% CFA. In experiment 2, BALB/c mice were immunized five times, first with 3.0 μg of gD-1 (HF strain) or gD-2 (Savage strain) then twice with 1.0 μg of gD-1 or gD-2 emulsified in 50% CFA, once with 0.5 μg of gD-1 or gD-2 emulsified in 50% CFA, and once with 0.5 μg of gD-1 or gD-2 in 0.15 M NaCl. All immunizations were given at biweekly intervals by intraperitoneal injection. The purification of gD-1 and gD-2 by means of an affinity column containing monoclonal reagents has been detailed previously (9). Control mice were sham immunized with CFA or 0.15 M NaCl alone. Seven days after the final injection in experiment 1, serum was obtained from each animal, and individual samples were tested for virus-neutralizing antibodies (4, 5, 9). In experiment 2, similar procedures were followed, except that serum samples from each immunization group were pooled before assay (see legend to Fig. 1 for SDS-PAGE analysis of pooled serum samples in experiment 2). Two months after the challenge, serum samples were obtained from surviving animals in experiment 2, pooled, and retested by immunoprecipitation and SDS-PAGE (8, 9, 26).

[c] Mouse groups were challenged intraperitoneally with HSV 14 days after final immunization or sham immunization as indicated above. A dosage of four 50% lethal doses was previously determined to yield 100% killing of sham-immunized BALB/c mice for both HSV-1 (Patton strain, 4×10^6 PFU) and HSV-2 (186 strain, 1×10^6 PFU) within 7 to 10 days and was used for experiment 1. A dosage of 20 50% lethal doses of HSV-2 (186 strain, 5×10^6 PFU) was employed for experiment 2.

[d] One animal died on day 1 after challenge.

challenge by either the homologous or heterologous virus. It should also be noted that the homologous virus used for challenge was a strain of HSV-1 different that used as the source of gD-1.

The presence of survivors in the CFA-sham-immunized group led us to increase the challenge dose of virus in the next experiment and to sham immunize all of the control animals with 50% CFA. In this experiment, animals were immunized with 6 μg of either gD-1 or gD-2. Serum samples from mice in each of the immunization groups were again obtained at the end of the immunization course, pooled, and assayed for neutralizing antibody and gD specificity. The neutralization titers ranged from 1:128 to 1:512. None of the CFA-sham-immunized mice possessed neutralizing antibodies against HSV.

The gD specificity of the pooled antisera is demonstrated in Fig. 1. The cell extract used to assess the antibodies present in the serum samples was prepared from HSV-1- or HSV-2-infected cells that were labeled with [^{3}H]arginine for 15 min at 6 h postinfection. We had previously shown that under these conditions, the precursor forms of gD (pgD-1 and pgD-2) as well as other viral glycoproteins were extensively labeled (4, 8, 9). Lanes 1 and 2 of Fig. 1 represent control immunoprecipitations of HSV-1- and HSV-2-infected cell extracts, respectively, made by using a previously prepared monospecific anti-gD-1 serum (4, 9). Lanes 3 and 4 represent immunoprecipitations of HSV-1- and HSV-2-infected cell extracts carried out with a pooled serum sample taken from mice immunized with gD-1. Lanes 5 and 6 represent a similar immunoprecipitation carried out with a pooled serum sample obtained from mice immunized with gD-2. Lanes 7 and 8 represent immunoprecipitations made by using a pooled serum sample obtained from sham-immunized mice. The results show that animals immunized with gD-1 or gD-2 responded by producing gD-specific antibodies. These data also confirm the purity of the gD-1 and gD-2 preparations used for immunization and further document the cross-reactivity of gD-1 and gD-2 antibodies (9).

Table 1 shows that none of the sham-immunized animals in experiment 2 survived the challenge. In contrast, all of the animals immunized with gD-2 and five out of six animals immunized with gD-1 survived the HSV-2 challenge. It should be noted that this one animal died within 1 day after challenge, whereas all of the sham-immunized animals died 5 to 8 days after challenge. It is thus possible that the one death among gD-immunized animals was due to reasons not directly related to a lack of protection. In any event, the results of the second experiment show that both glycoproteins appeared to be effective in protection against challenge by HSV-2. Similar experiments with affinity-purified gD-1 as the immunogen have been repeated independently; all 10 gD-1-immunized mice survived an HSV-2 challenge, whereas

1 2 3 4 5 6 7 8

FIG. 1. SDS-PAGE analysis of antisera to gD-1 and gD-2 obtained before virus challenge. Serum samples from immunization groups (Table 1, experiment 2) were obtained 7 days after completion of the immunization cycle and 7 days before challenge. Control samples or pooled samples from immunization groups were tested by immunoprecipitation of cytoplasmic extracts obtained from HSV-1- or HSV-2-infected cells that were pulse-labeled with [^{3}H]arginine for 15 min at 6 h postinfection (4, 8, 9). Lanes 1, 3, 5, and 7 contain extract from HSV-1-infected cells; lanes 2, 4, 6, 8 contain extract from HSV-2-infected cells. Lanes 1 and 2, Rabbit anti-gD-1 control (19); lanes 3 and 4, pooled serum from mice immunized with gD-1 in CFA (Table 2, experiment 2); lanes 5 and 6, pooled serum from mice immunized with gD-2 in CFA (experiment 2); lanes 7 and 8, serum from mice sham immunized with CFA (experiment 2).

none of 10 sham-immunized mice survived (G. Cerini, personal communication).

Two months after the challenge, serum samples were obtained from the surviving gD-1- and gD-2-immunized animals in experiment 2. Samples from each group were pooled and retested by immunoprecipitation and SDS-PAGE (Fig. 2). The serum samples continued to exhibit a major response to gD (compare Fig. 1 and 2) but also displayed an increased heterogeneity, in that antibodies to other viral components, including viral glycoproteins, were present after challenge. This heterogeneity might simply be a response to the infecting dose of virus, or it might be a response to virus which replicated after infection. Further experiments should be done to clarify this point.

The current investigations show that active immunization with gD purified from HSV-1- or HSV-2-infected cells confers protection against lethal challenge with both homologous and heterologous virus types. Moreover, the protective effect is correlated with the presence of gD-specific antibodies. Recovery from HSV infections appears to involve both humoral and cell-mediated immunity (2, 10, 13, 17, 21, 24, 29). It was recently shown that gC of HSV-1 induces type-specific protective immunity in the absence of detectable antibodies (23). However, studies showing conferral of protection after the passive transfer of monoclonal anti-gD antibodies (1, 6, 10, 22) are entirely consistent with the premise that antibodies to gD can exert a significant protective effect. Regardless of the immune mechanism responsible, it is clear from the present investigation that purified gD is an important candidate for a subunit vaccine potentially effective against herpetic infections. It remains to be seen whether such a subunit vaccine will protect against establishment of latency or recurrent infection.

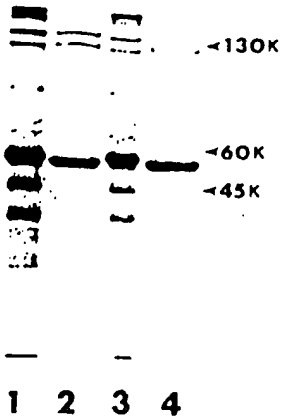

FIG. 2. SDS-PAGE analysis of pooled serum samples taken from immunized mice (Table 1, experiment 2) 2 months after challenge with HSV-2 strain 186. The cytoplasmic extracts are the same as those shown in Fig. 1. Lanes 1 and 3 contain extract from HSV-1-infected cells; lanes 2 and 4 contain extract from HSV-2-infected cells. Lanes 1 and 2, Pooled serum from animals immunized with gD-1; lanes 3 and 4, pooled serum from animals immunized with gD-2.

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EXHIBIT 7

United States Patent [19]

Kensil et al.

[11] Patent Number: 5,057,540

[45] Date of Patent: Oct. 15, 1991

[54] SAPONIN ADJUVANT

[75] Inventors: Charlotte A. Kensil, Milford; Dante J. Marciani, Hopkinton, both of Mass.

[73] Assignee: Cambridge Biotech Corporation, Worcester, Mass.

[21] Appl. No.: 573,268

[22] Filed: Aug. 27, 1990

Related U.S. Application Data

[63] Continuation of Ser. No. 200,754, May 31, 1988, abandoned, which is a continuation-in-part of Ser. No. 55,229, May 29, 1987, abandoned.

[51] Int. Cl.[5] A61K 31/70; A61K 31/705; A61K 39/00

[52] U.S. Cl. 514/25; 514/26; 514/33; 514/35; 514/885; 424/88; 424/195.1; 536/4.1; 536/6.3; 536/5

[58] Field of Search 514/25, 26, 33, 35, 514/885; 424/88, 89, 195.1; 536/4.1, 6.3, 18.1, 127, 128, 5

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Mostad and Doehl, *J. of Chromatography*, 396:157-168 (1987).

Egerton et al., *Vet. Scl. Comm.*, 2:247-252 (1978).

McColm et al., *Parasite Immun.*, 4:337-347 (1982).

Kartnig et al., *Planta Medica*, 23(3):269-271 (1973).

Primary Examiner—Ronald W. Griffin

Assistant Examiner—Nancy S. Carson

Attorney, Agent, or Firm—Sterne, Kessler, Goldstein & Fox

[57] ABSTRACT

Substantially pure saponins are disclosed. The saponins of the present invention are useful as immune adjuvants. Disclosed as well are immune response-provoking compositions comprising an antigen in admixture with the substantially pure saponins.

16 Claims, 23 Drawing Sheets

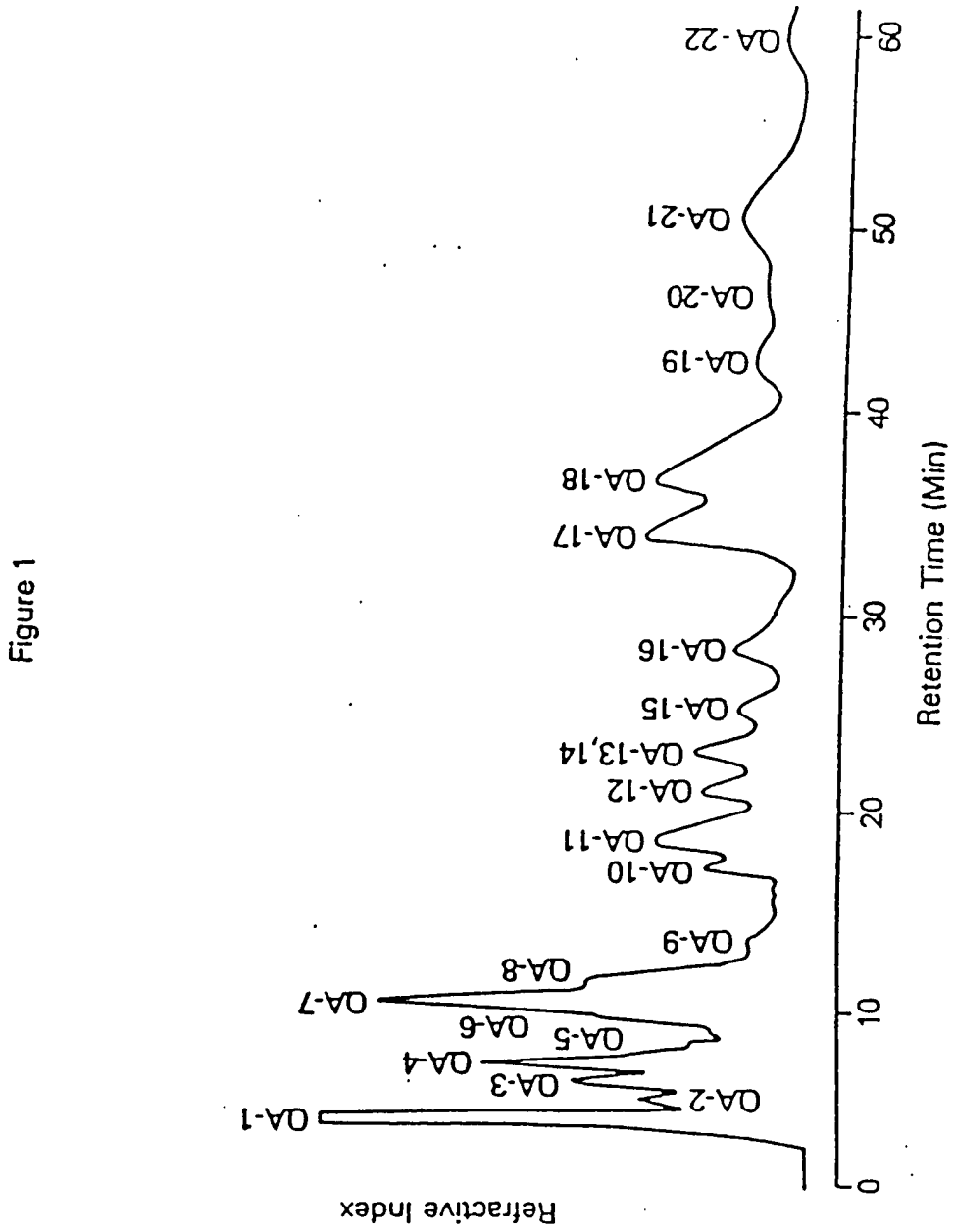
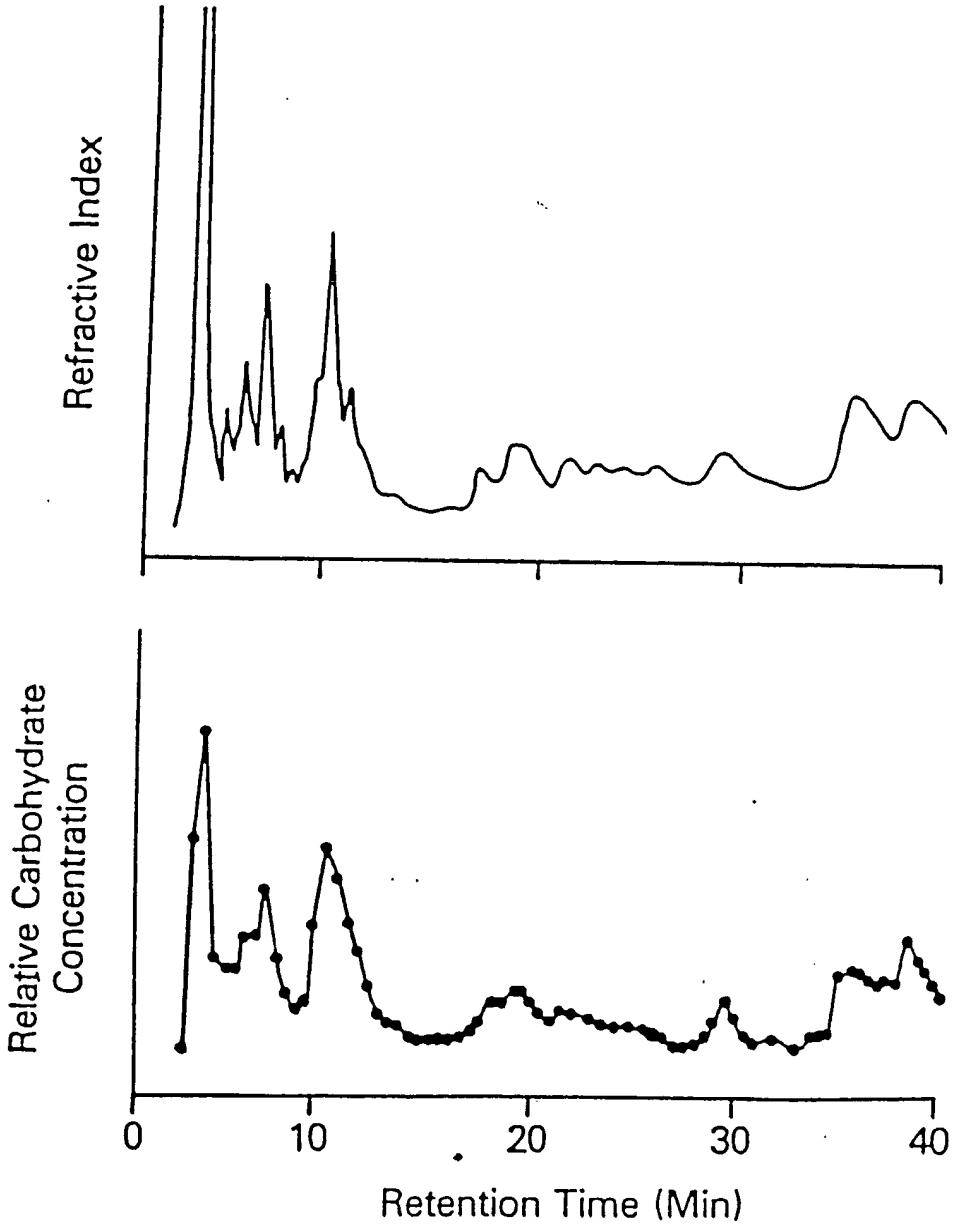

OA-1
OA-2
OA-3
OA-4
OA-5
OA-6
OA-7
OA-8
OA-9
OA-10
OA-11
OA-12
OA-13,14
OA-15
OA-16
OA-17
OA-18
OA-19
OA-20
OA-21
OA-22
Refractive Index
0
10
20
30
40
50
60
Retention Time (Min)

Figure 1

Figure 2


Refractive Index
Relative Carbohydrate Concentration
0
10
20
30
40
Retention Time (Min)

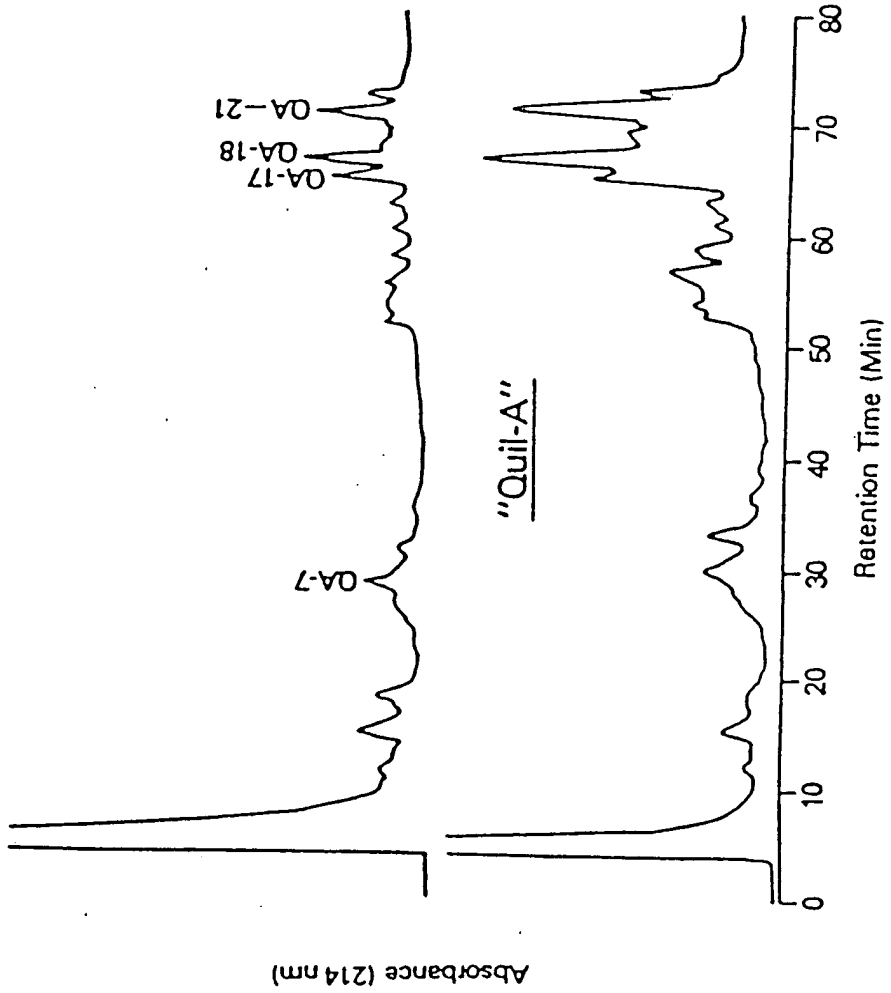
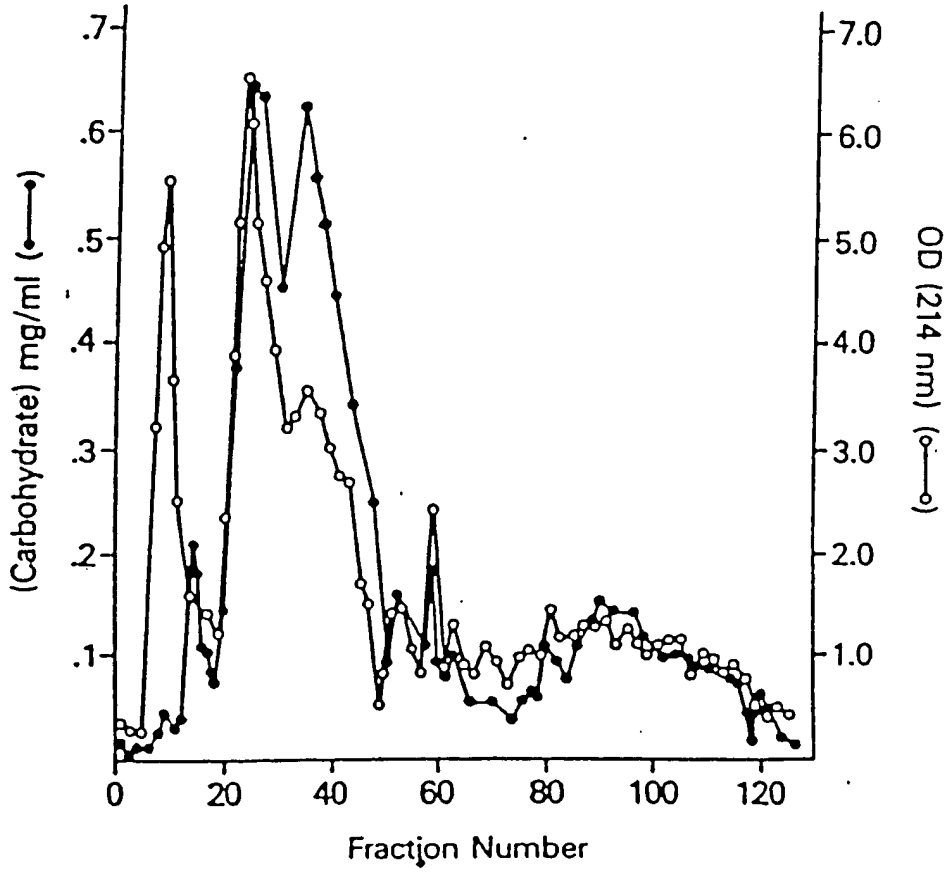

QA-21
QA-18
QA-17
QA-7
"Quil-A"
0
10
20
30
40
50
60
70
80
Retention Time (Min)
Absorbance (214 nm)

Figure 3

Figure 4A


.7
.6
.5
.4
.3
.2
.1
(Carbohydrate) mg/ml (●—●)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
OD (214 nm) (○—○)
0
20
40
60
80
100
120
Fraction Number

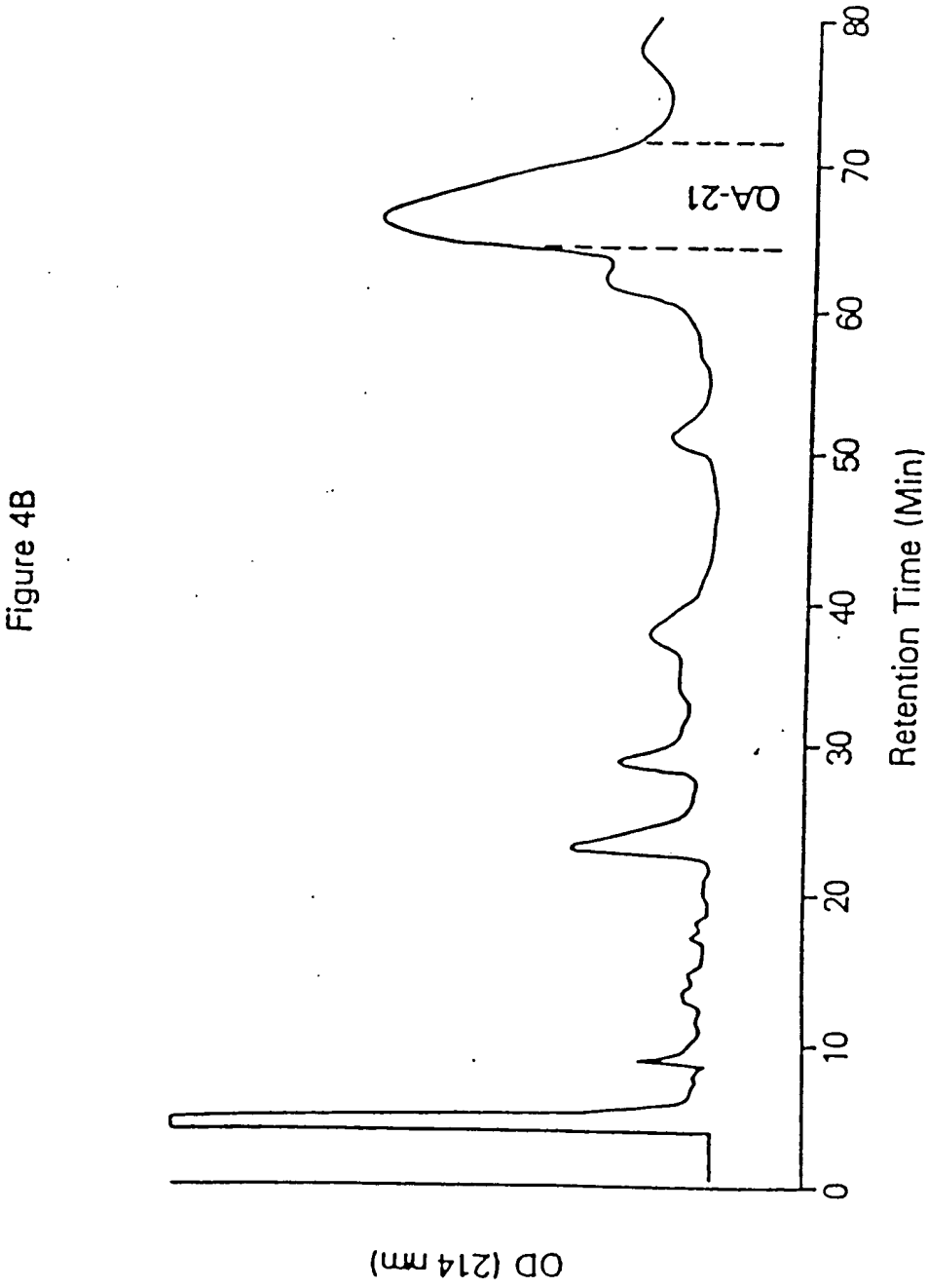


Figure 4B

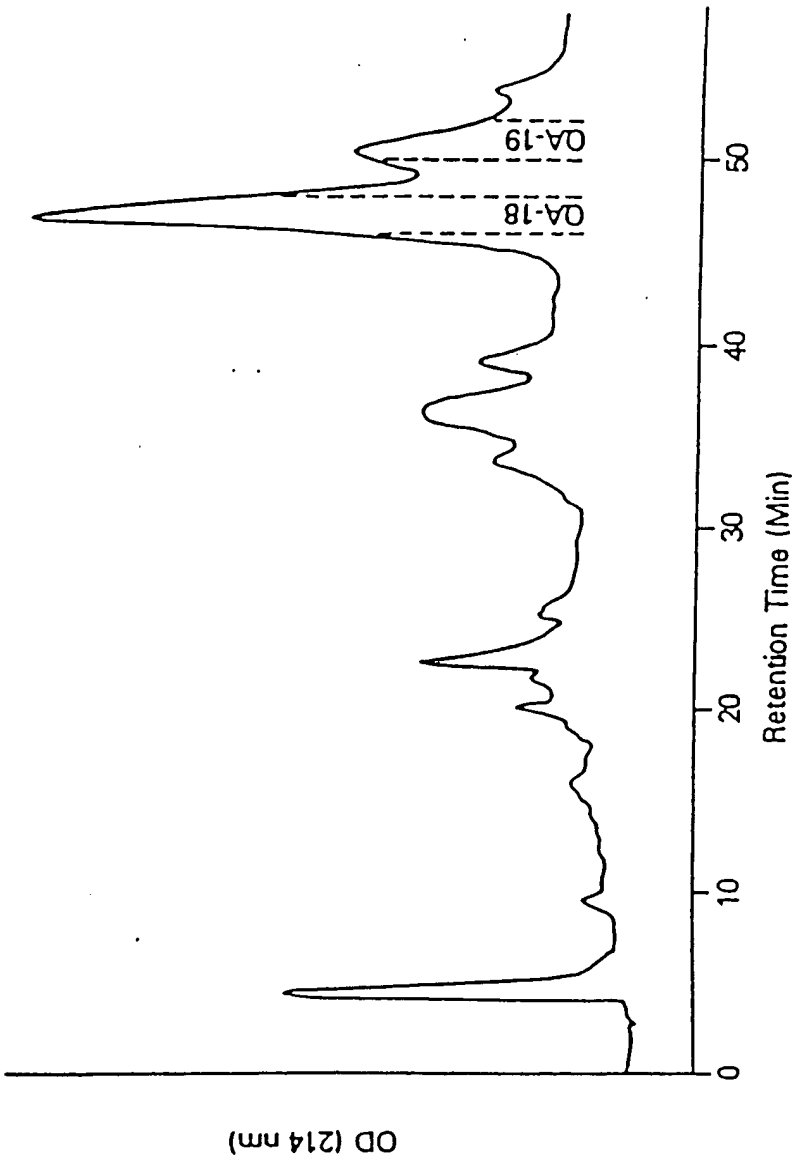


Figure 4C

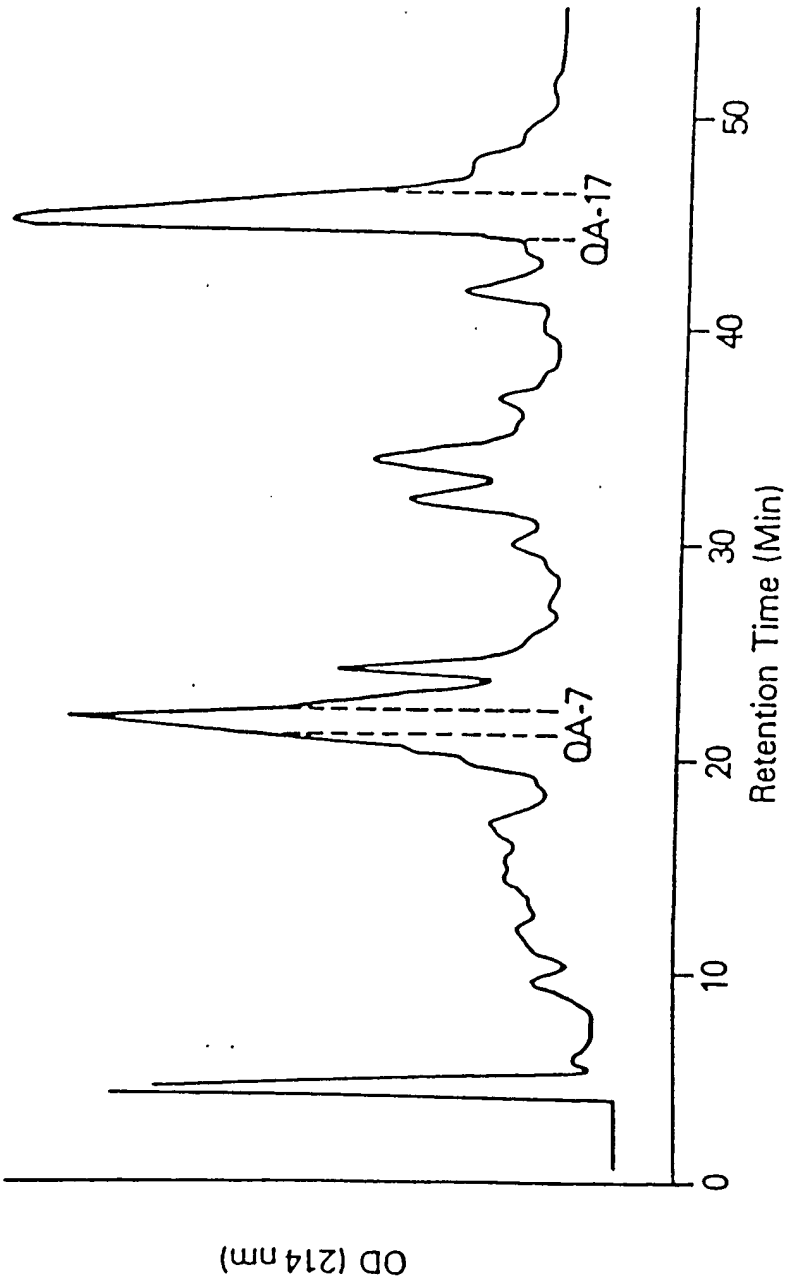


Figure 4D

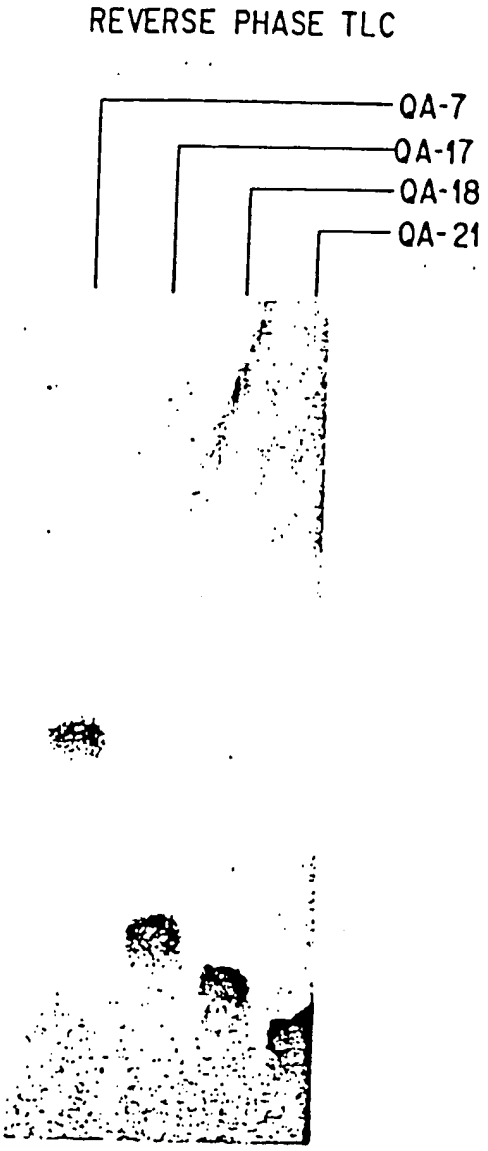


FIG. 5A

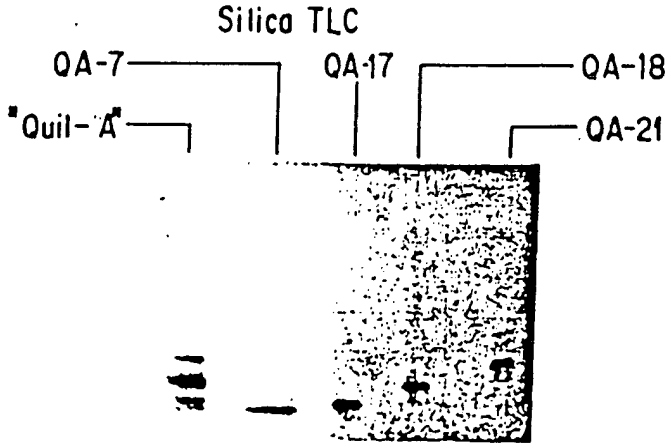
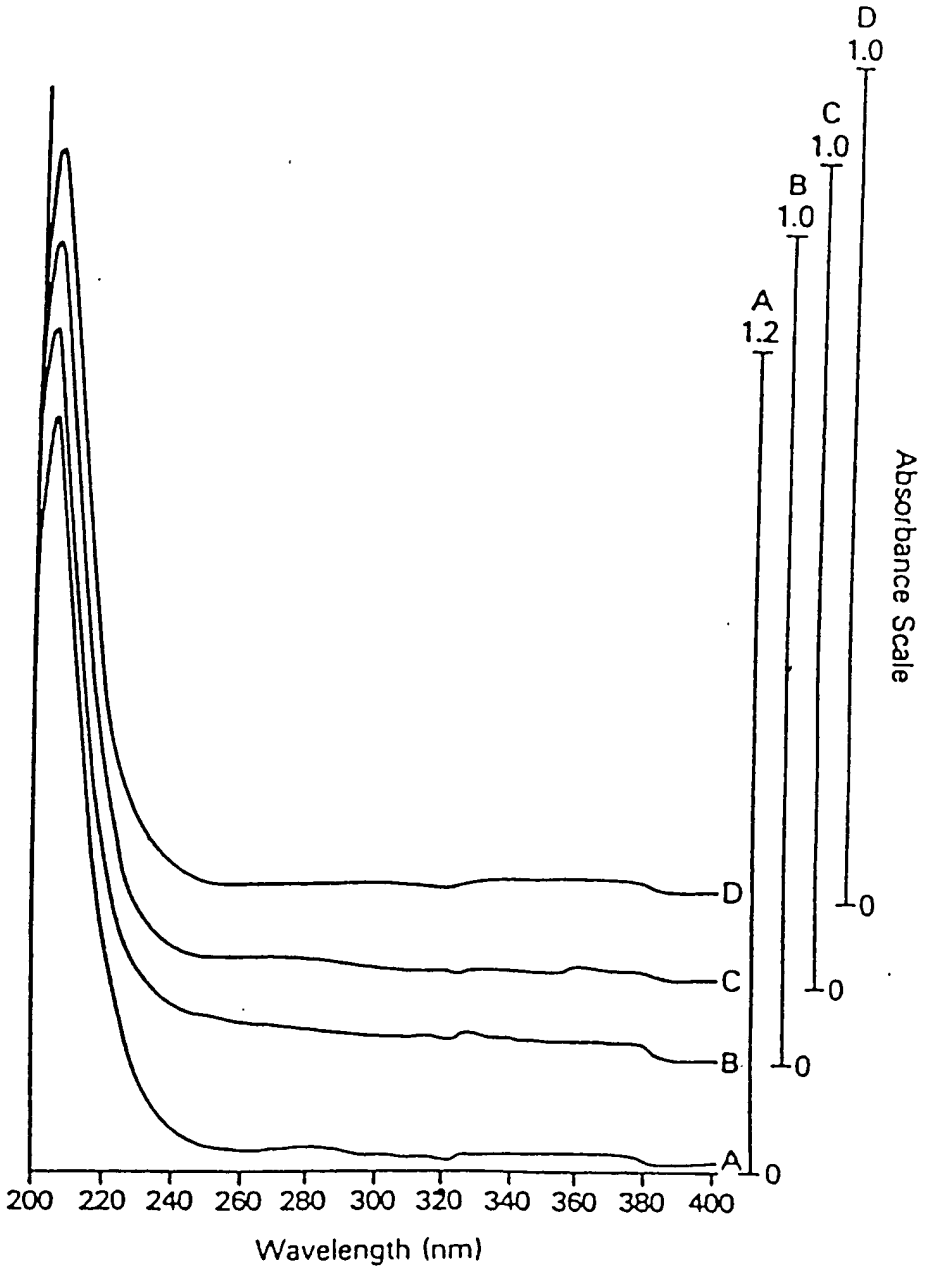


FIG. 5B

Figure 6


D
1.0
C
1.0
B
1.0
A
1.2
Absorbance Scale
D
C
B
A
0
0
0
0
200 220 240 260 280 300 320 340 360 380 400
Wavelength (nm)

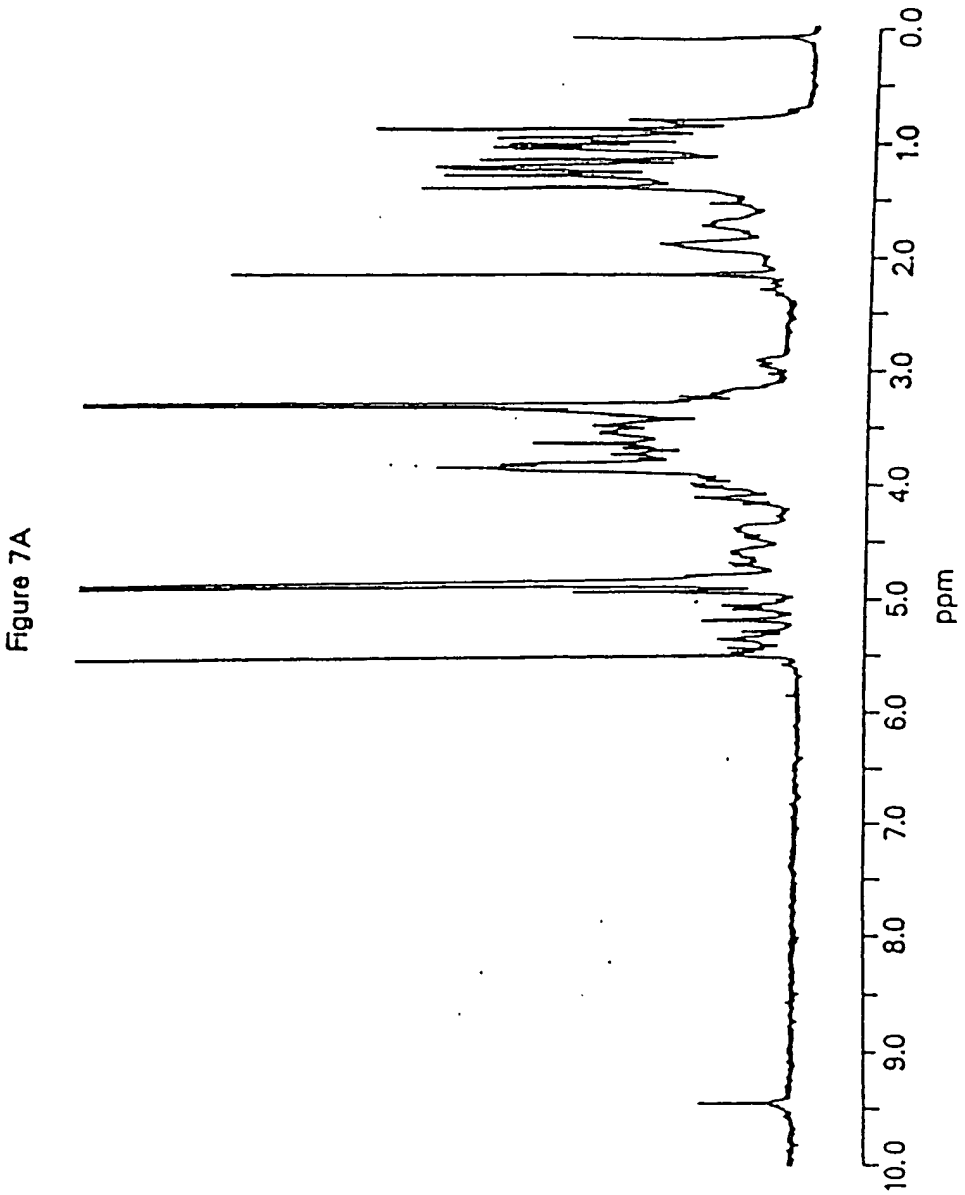


Figure 7A

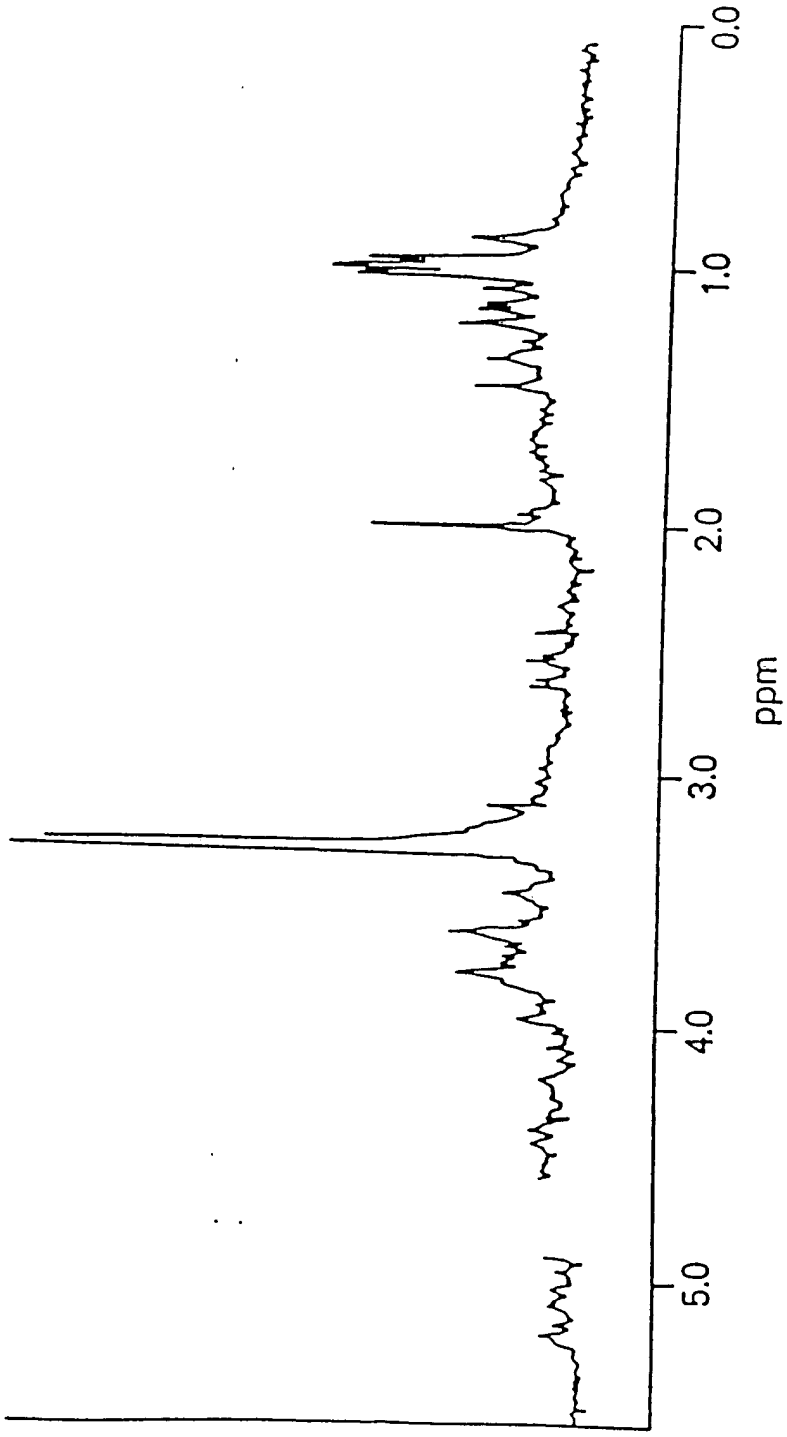


Figure 7B

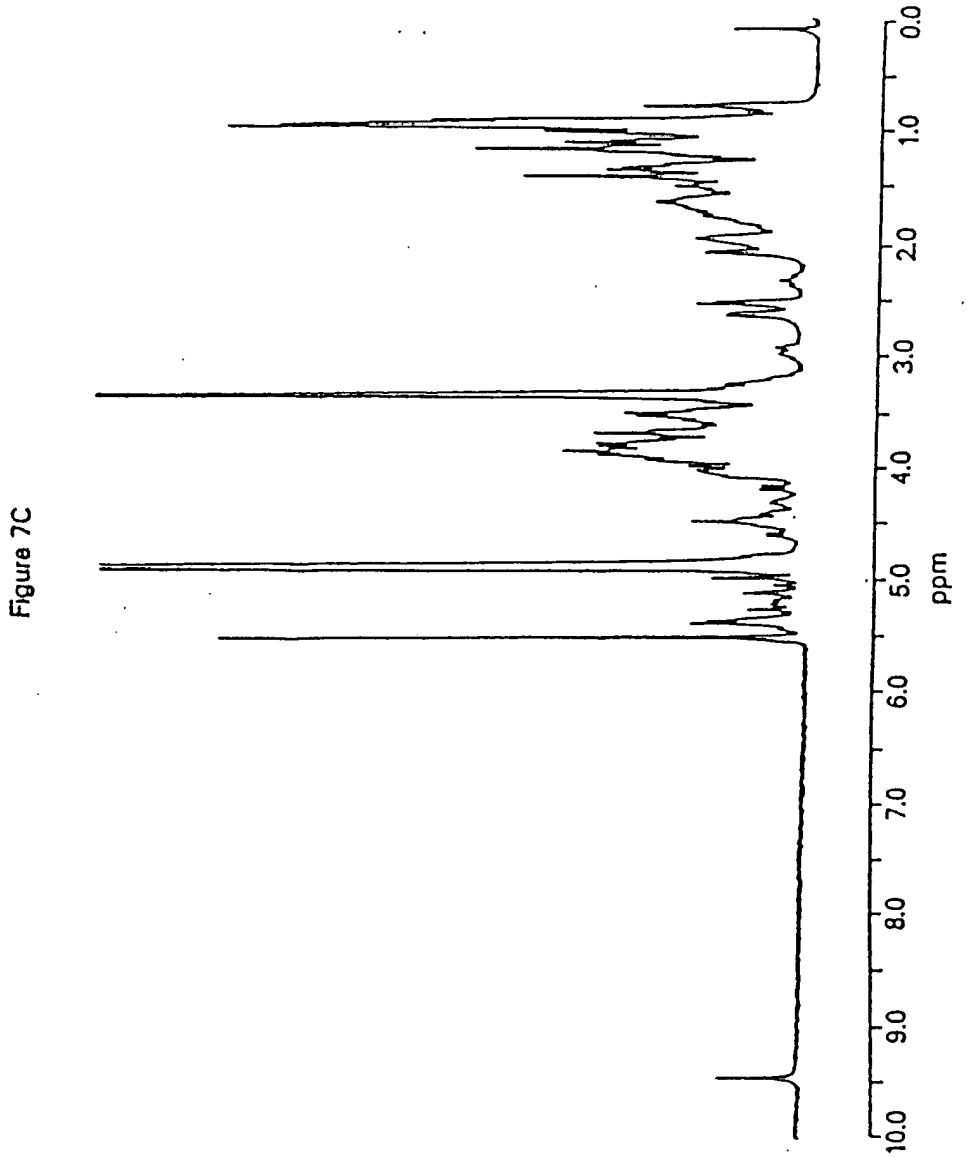


Figure 7C

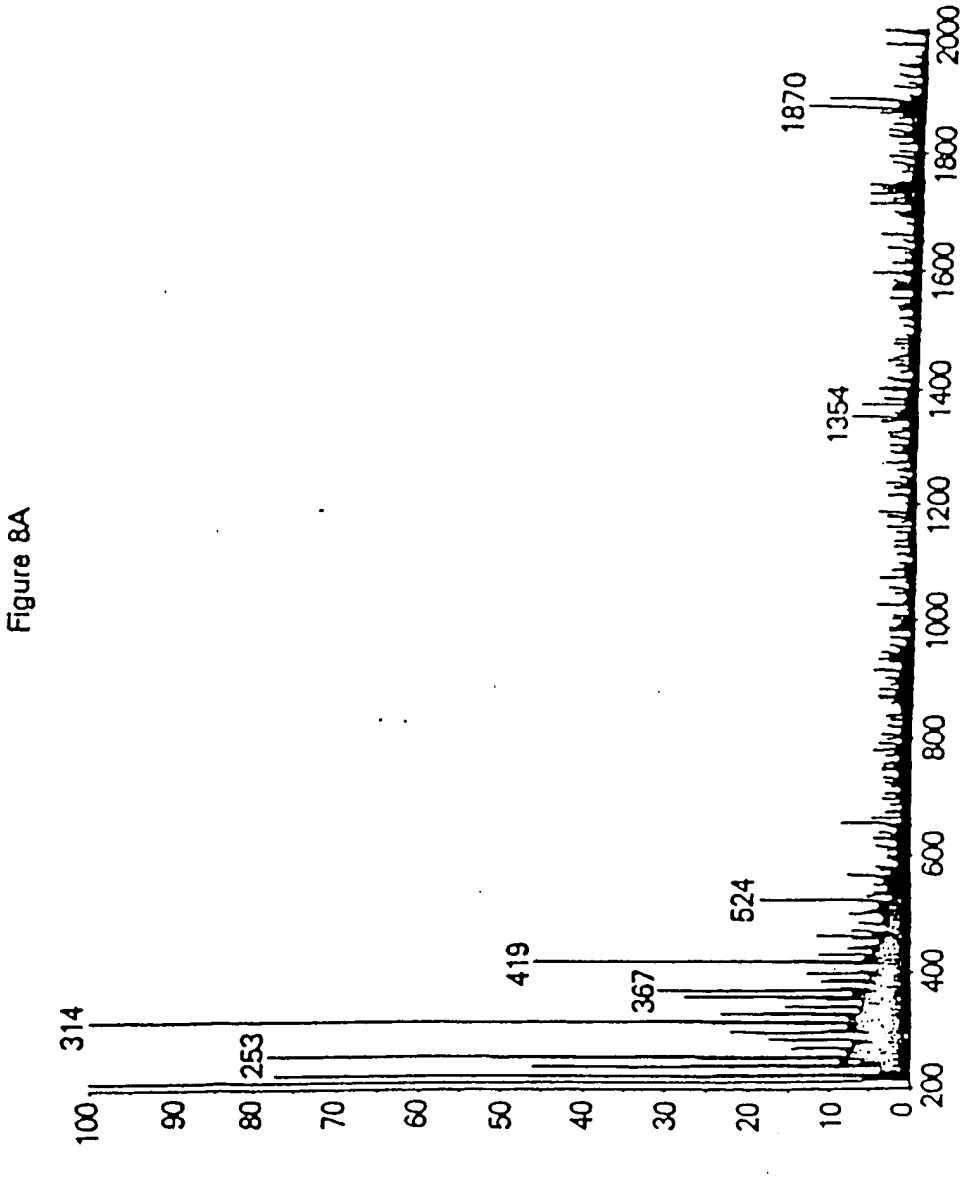

314
253
419
367
524
1354
1870
100
90
80
70
60
50
40
30
20
10
0
200
400
600
800
1000
1200
1400
1600
1800
2000

Figure 8A

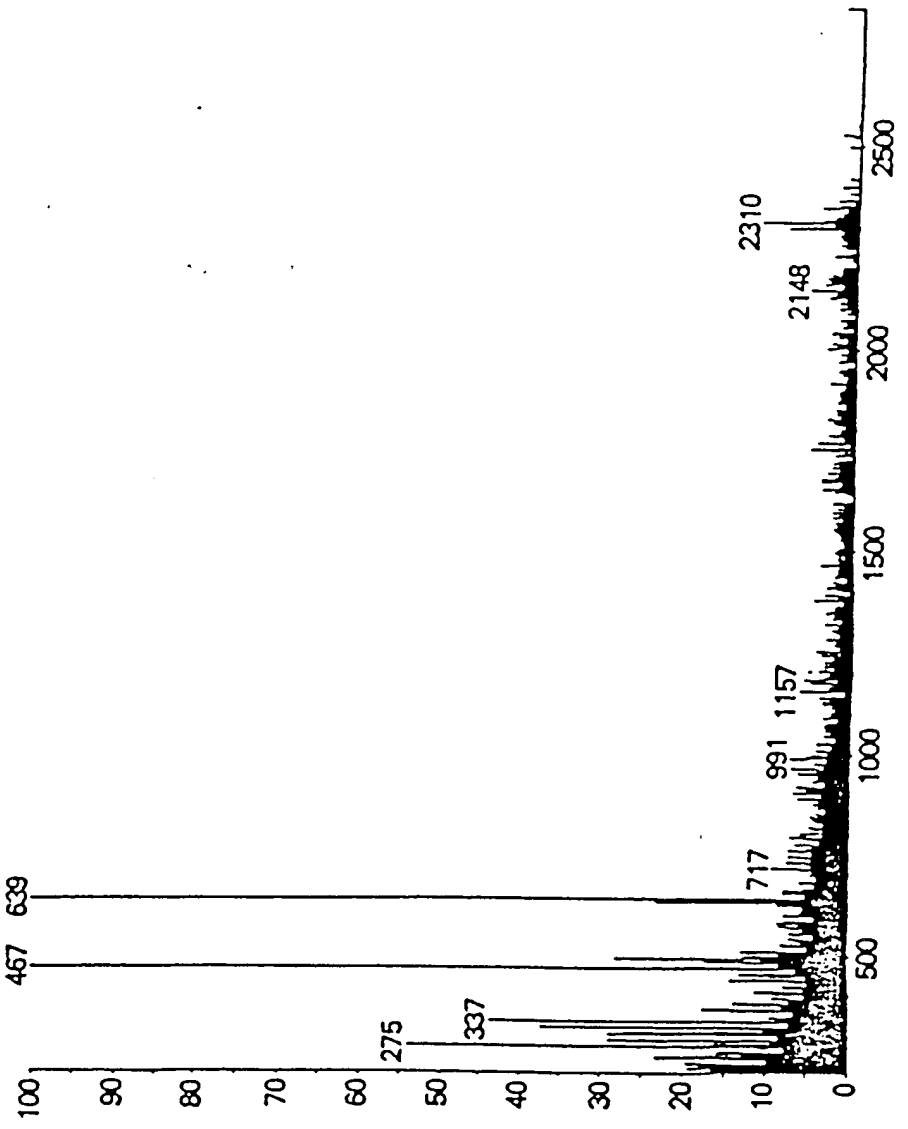

639
467
275
337
717
991
1157
2310
2148
100
90
80
70
60
50
40
30
20
10
0
500
1000
1500
2000
2500

Figure 8B

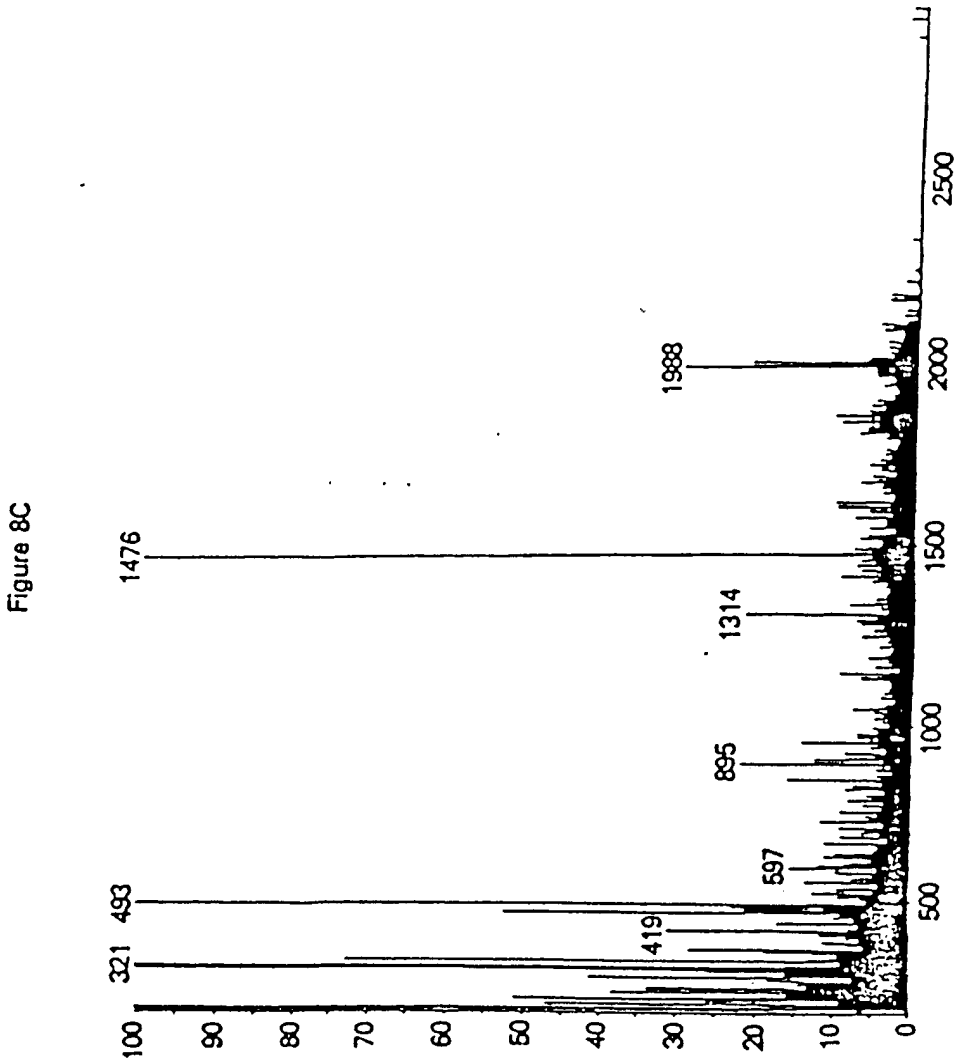

100
90
80
70
60
50
40
30
20
10
0
500
1000
1500
2000
2500
493
321
419
597
895
1314
1476
1988

Figure 8C

Figure 9

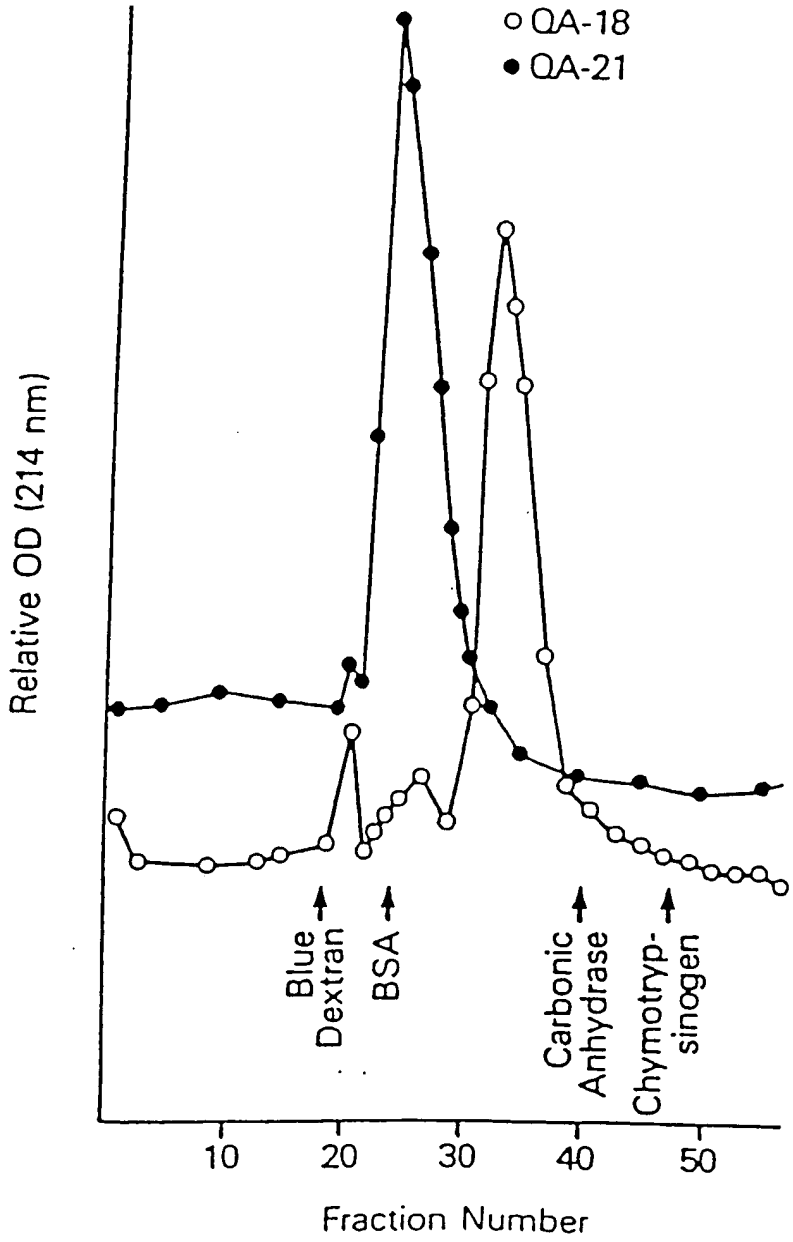
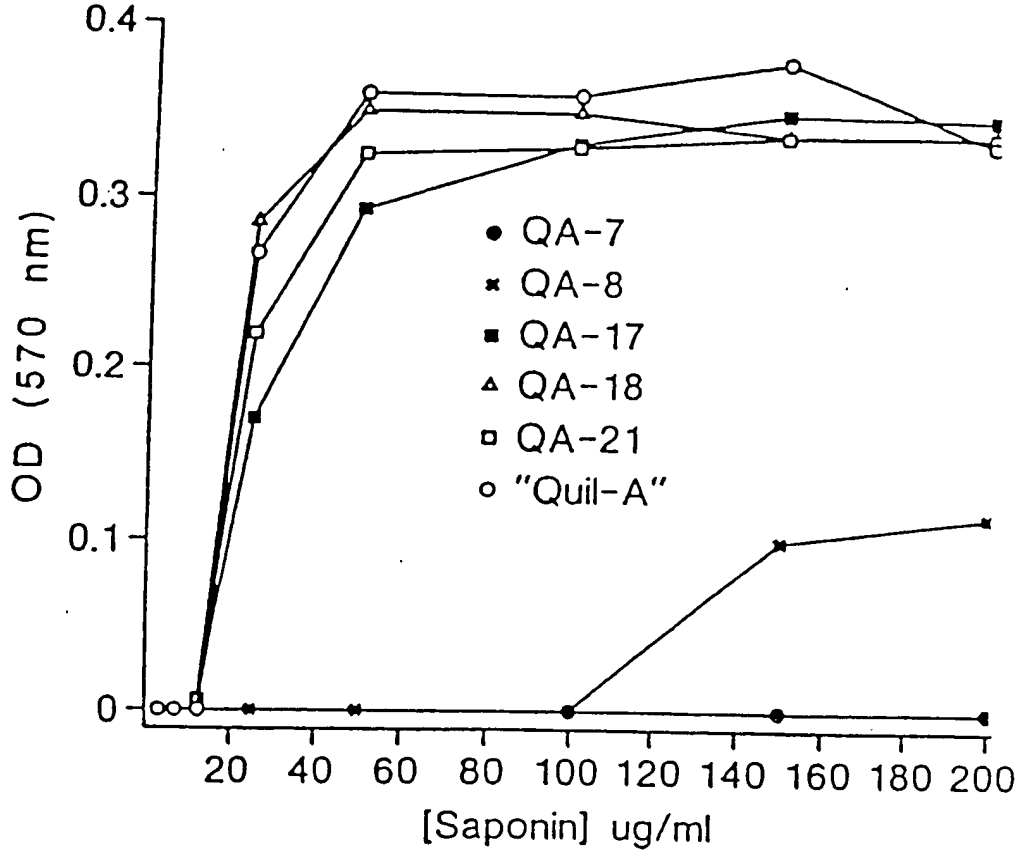

QA-18
QA-21
Relative OD (214 nm)
Blue Dextran
BSA
Carbonic Anhydrase
Chymotryp-sinogen
10
20
30
40
50
Fraction Number

Figure 10


0.4
0.3
0.2
0.1
0
OD (570 nm)
20 40 60 80 100 120 140 160 180 200
[Saponin] ug/ml
QA-7
QA-8
QA-17
QA-18
QA-21
"Quil-A"

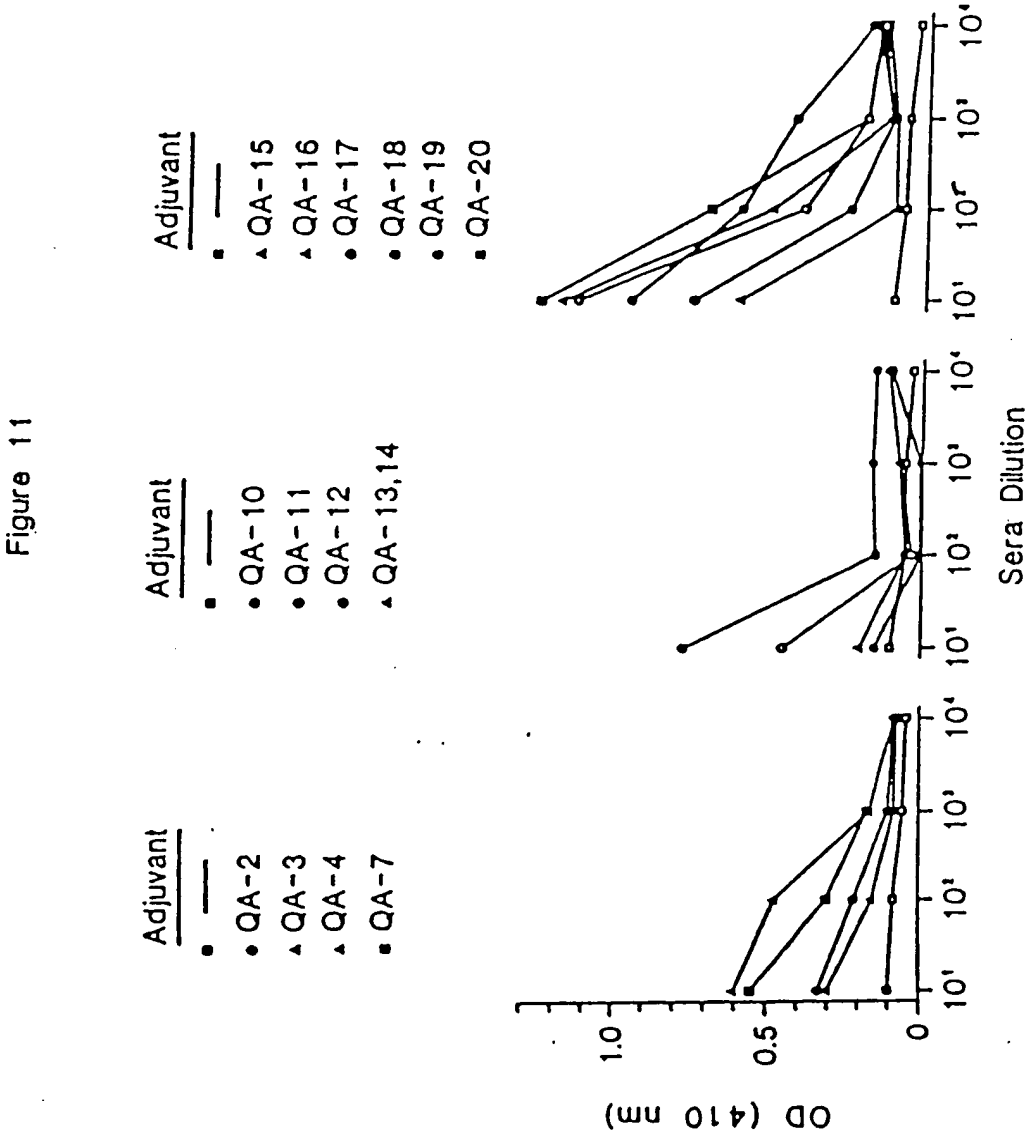

Adjuvant
— —
• QA-2
▲ QA-3
▲ QA-4
■ QA-7
Adjuvant
— —
• QA-10
• QA-11
• QA-12
▲ QA-13,14
Adjuvant
— —
▲ QA-15
▲ QA-16
• QA-17
• QA-18
• QA-19
■ QA-20
OD (410 nm)
1.0
0.5
0
10¹
10²
10³
10⁴
Sera Dilution

Figure 11

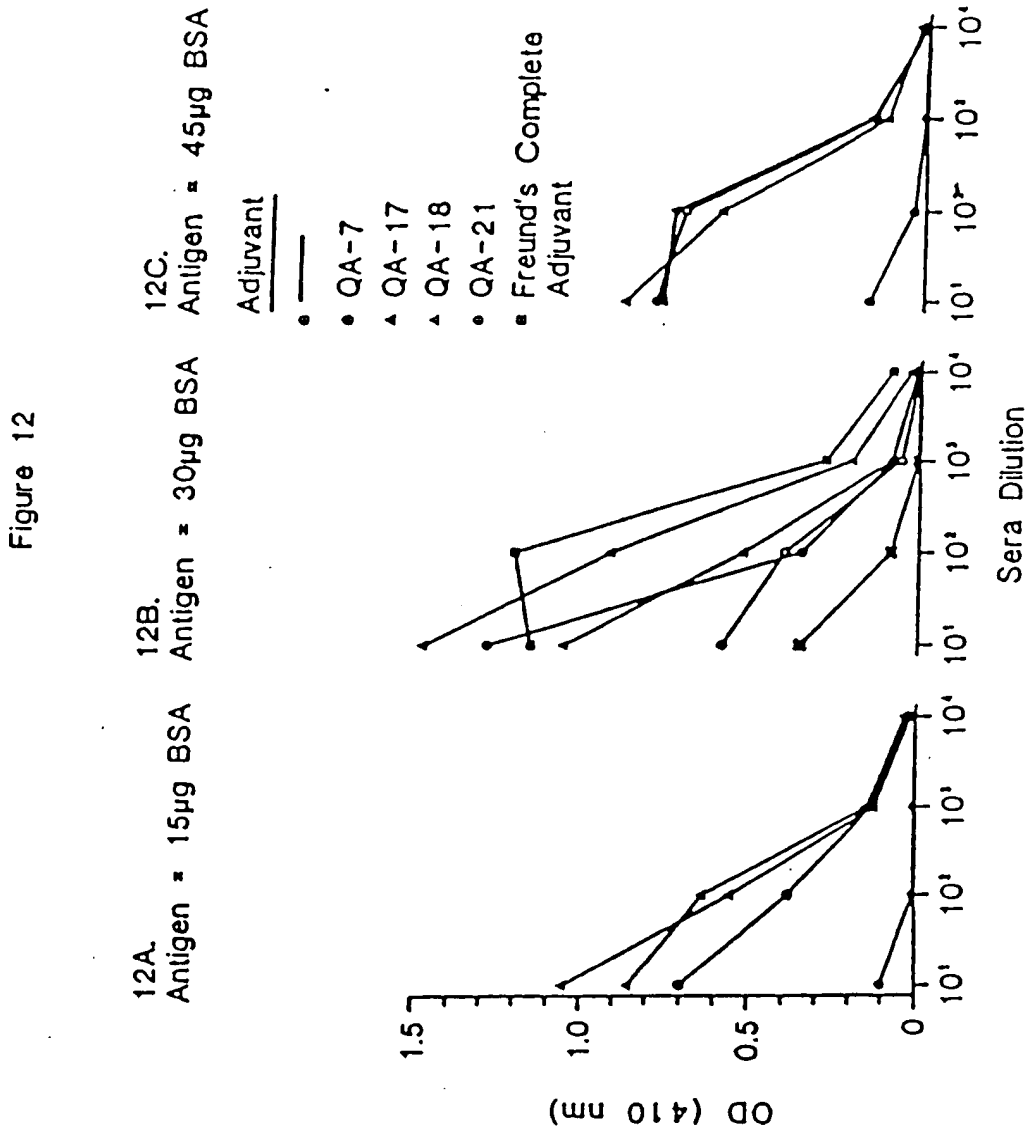

12A.
Antigen = 15µg BSA
12B.
Antigen = 30µg BSA
12C.
Antigen = 45µg BSA
Adjuvant
—
QA-7
QA-17
QA-18
QA-21
Freund's Complete Adjuvant
OD (410 nm)
1.5
1.0
0.5
0
10¹
10²
10³
10⁴
Sera Dilution

Figure 12

Figure 13

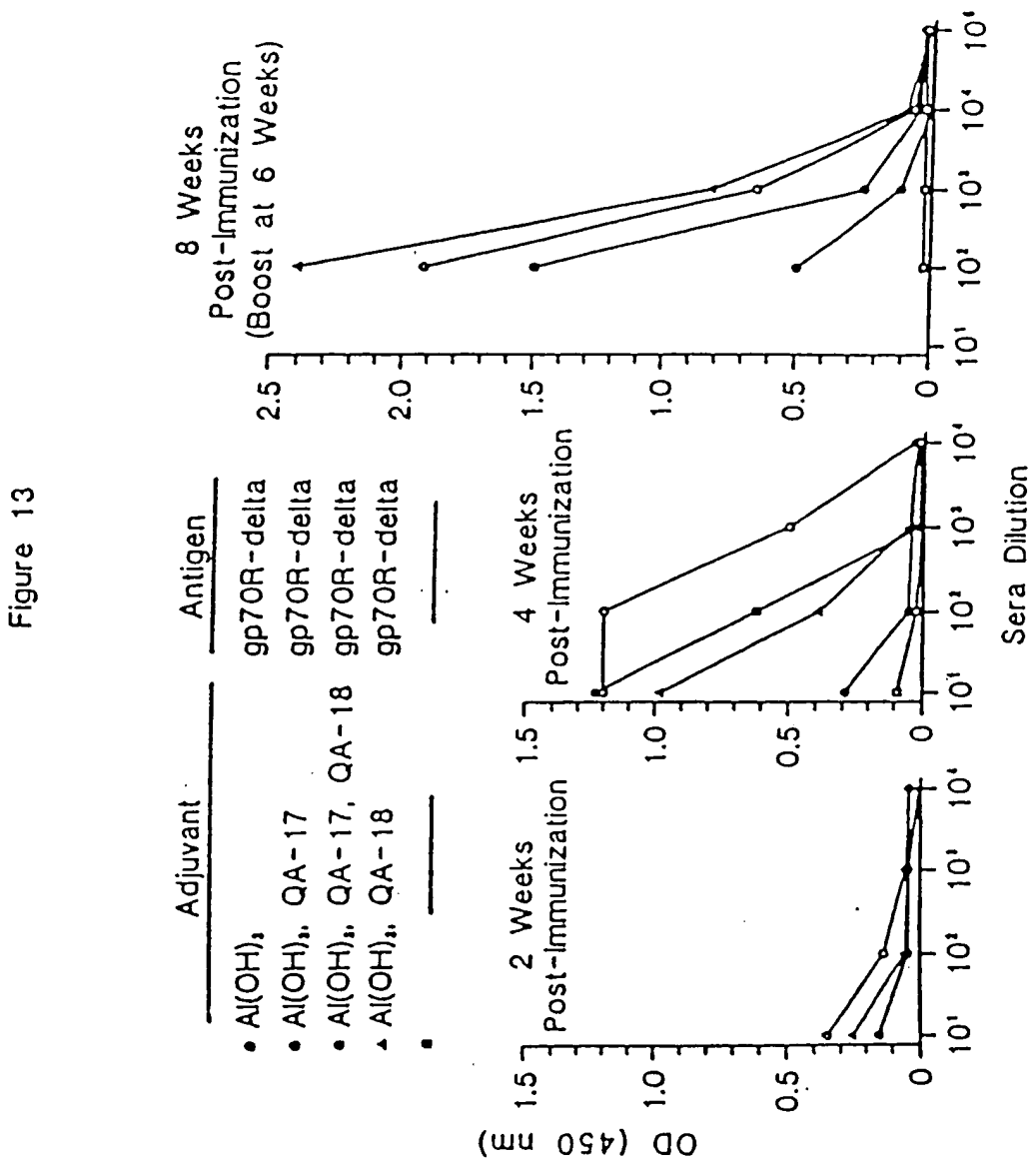

Adjuvant
Antigen
• Al(OH)3
gp70R-delta
• Al(OH)3, QA-17
gp70R-delta
• Al(OH)3, QA-17, QA-18
gp70R-delta
▲ Al(OH)3, QA-18
gp70R-delta
2 Weeks Post-Immunization
4 Weeks Post-Immunization
8 Weeks Post-Immunization (Boost at 6 Weeks)
OD (450 nm)
Sera Dilution

Figure 14

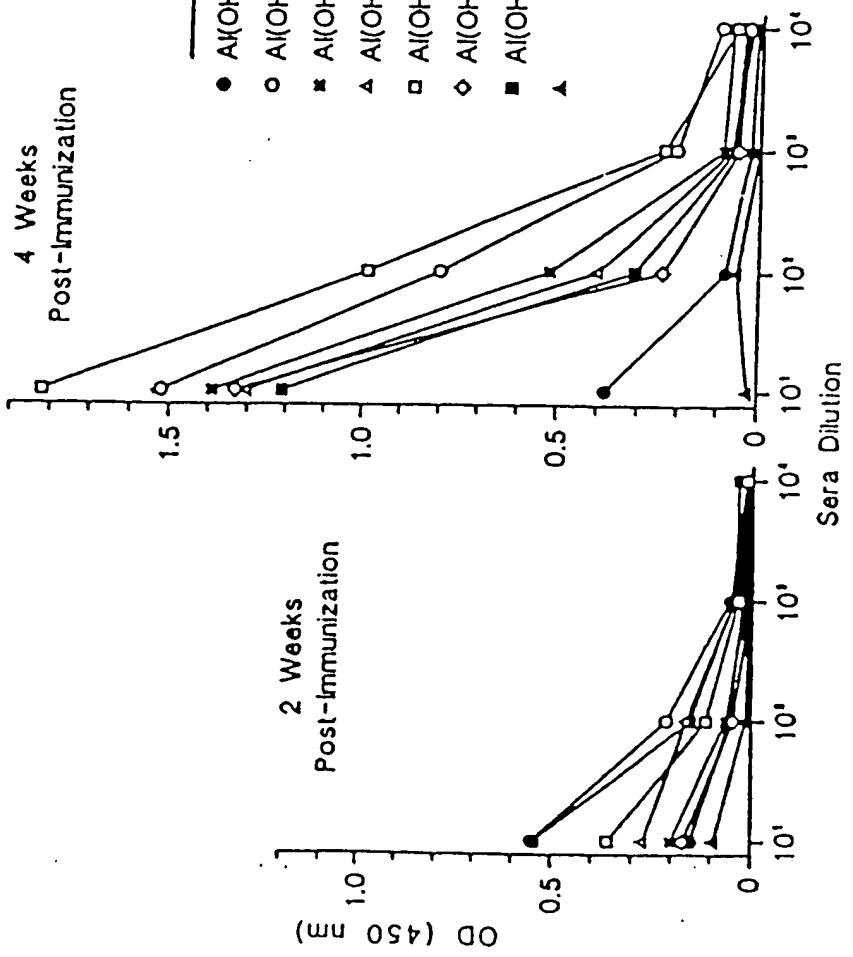
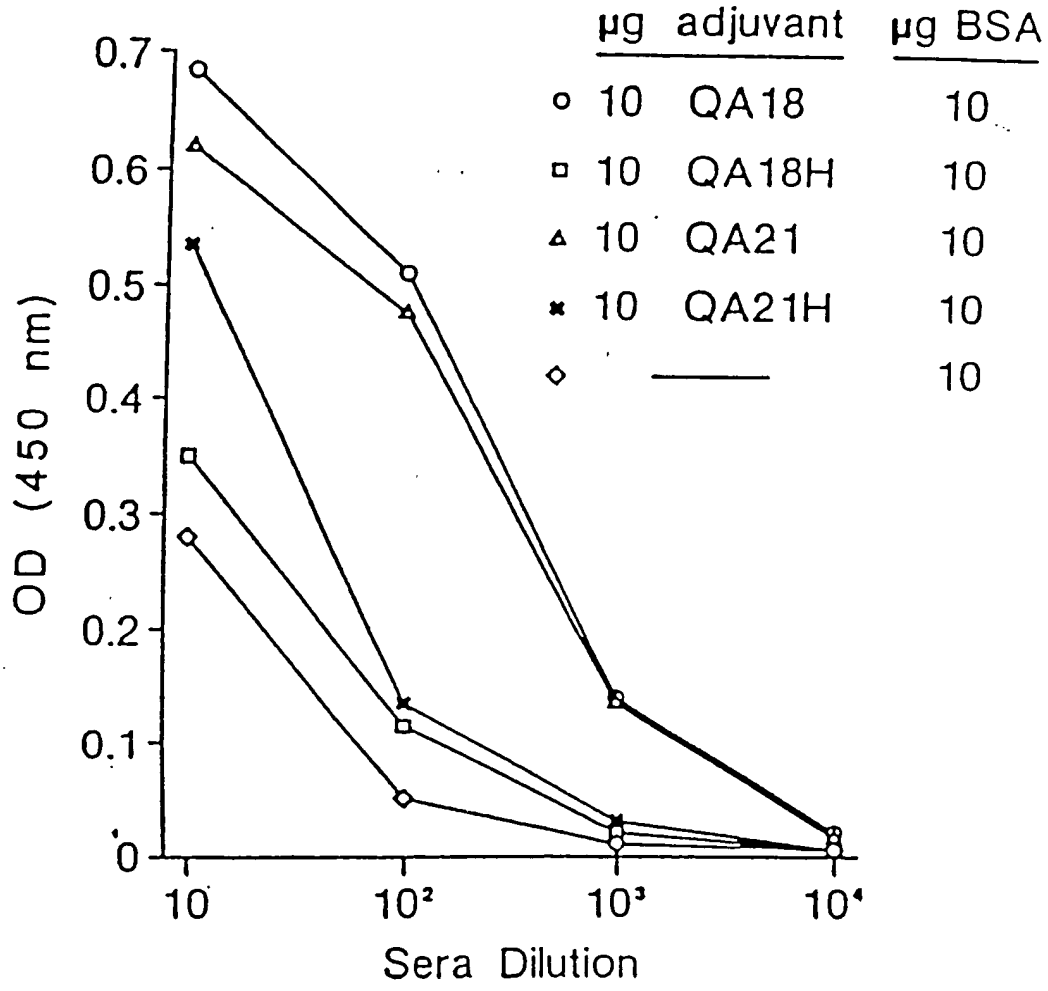

Adjuvant
Antigen
Al(OH)3
Alkylated gp70R-del1
Al(OH)3, QA-17
Alkylated gp70R-del1
Al(OH)3, QA-18
Alkylated gp70R-del1
Al(OH)3, QA-7
Alkylated gp70R-del1
Al(OH)3, QA-17, QA-18
Alkylated gp70R-del1
Al(OH)3, QA-7, QA-17
Alkylated gp70R-del1
Al(OH)3, QA-7, QA-18
Alkylated gp70R-del1
4 Weeks Post-Immunization
2 Weeks Post-Immunization
OD (450 nm)
Sera Dilution

Figure 15


µg adjuvant
µg BSA
10 QA18 10
10 QA18H 10
10 QA21 10
10 QA21H 10
10
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD (450 nm)
10
10²
10³
10⁴
Sera Dilution

SAPONIN ADJUVANT

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of application Ser. No. 07/200,754, filed 05/31/88 which is a continuation-in-part of U.S. patent application Ser. No. 055,229 filed May 29, 1987 and having the title "Saponin Adjuvant" both now abandoned.

This application is also related to U.S. patent application Ser. No. 55,298, which is a continuation-in-part of U.S. patent application Ser. No. 868,585, entitled "Method of Preparation and Use For Feline Leukemia Virus Antigens," in the names of Beltz et al.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the field of immune adjuvants, the process for production thereof, and the use thereof as immune adjuvants and vaccines.

2. Brief Description of the Background Art

Quillaja saponins are a mixture of triterpene glycosides extracted from the bark of the tree *Quillaja saponaria*. Crude saponins have been extensively employed as adjuvants in vaccines against foot and mouth disease, and in amplifying the protective immunity conferred by experimental vaccines against protozoal parasites such as *Trypanosoma cruzi* plasmodium and also the humoral response to sheep red blood cells (SRBC). (Bomford, *Int. Arch. Allerg. appl. Immun.*, 67:127 (1982)).

Saponins are natural products which have been characterized by a number of common properties. The ability to produce foam in aqueous solution gave the name to the group. Further characteristics are the hemolytic activity, the toxicity for fish, the complexing with cholesterol, and in some cases antibiotic activity. Kofler, *Die Saponine* (Springer Berlag), Berlin, 1927; Tschesche et al., *Chemine und Biologic der Saponine. Fortscher. Chem. Oro. Naturst.* XXX:461 (1972).

The common properties of saponins are not reflected in a common chemical composition. Although all saponins are glycosides, the aglycone may belong to the steroids, the triterpenoids, or the steroidalcaloids. The number of sugar and sugar chains attached to the glycosidic bonds may vary greatly. Saponins have been produced commercially and have many uses. The commercially available Quillaja saponins are crude mixtures which, because of their variability, are not desirable for use in veterinary practice or in pharmaceutical compositions for man. Because of the variability and heterogeneity, each batch must be tested in animal experiments to determine adjuvant activity and toxicity. The impurities in the commercially available products may produce adverse reactions. In addition, the content of the active substance in a given batch of saponin may vary, thereby decreasing the reproducibility from batch to batch.

An early attempt to purify Quillaja saponin adjuvants was made by Dalsgaard, *Archiv fuer die gesamte Virusforschung* 44:243 (1974). Dalsgaard partially purified an aqueous extract of the saponin adjuvant material from *Quillaja saponaria Molina*. Dalsgaard's preparation, commercially available from Superfos under the name "Quil-A," has been isolated from the bark of the South American tree, *Quillaja saponaria Molina*, and is characterized chemically as a carbohydrate moiety in glycosidic linkage to the triterpenoid quillaic acid. However, while the saponin Quil A of Dalsgaard presents a definite improvement over the previously available commercial saponins, it also shows considerable heterogeneity.

Higuchi et al., *Phytochemistry* 26:229 (January, 1987) treated a crude Quillaja saponin mixture with alkaline hydrolysis in 6% NH_4HCO_3 in 50% methanol and generated two major desacylsaponins, termed DS-1 and DS-2. DS-1 was shown to contain glucuronic acid, galactose, xylose, fucose, rhamnose, apiose, and Quillajic acid, whereas DS-2 contained these same components plus an additional glucose. Byproducts of this deacylation produced multiple components including 3,5-dihydroxy-6-methyloctanoic acid, 3,5-dihydroxy-6-methyloctanic acid, 5-α-L-arabinofuranoside and 5-O-α-L-rhamnopyranosyl-(1->2)-α-L-arabinofuranoside (Higuchi et al., *Phytochemistry* 26:2357 (August, 1987).

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the refractive index profile of dialyzed, methanol-solubilized Quillaja bark extract on reverse phase-HPLC.

FIG. 2 shows that the refractive index peaks of the above sample correspond to carbohydrate peaks.

FIG. 3 shows the comparison of Superfos "Quil-A" and dialyzed methanol soluble bark extract by HPLC.

FIG. 4 shows the purification of QA-7, QA-17, QA-18, QA-19, and QA-21 from "Quil-A," a crude saponin mixture, by silica chromatography (4A) and subsequent reverse phase chromatography (4B, 4C, 4D).

FIG. 5 demonstrates the purity of QA-7, QA-17, QA-18, and QA-21 by reverse phase (5A) and normal phase (5B) thin layer chromatography.

FIG. 6A shows the UV spectrum of QA-7. FIG. 6B shows the UV spectrum of QA-17. FIG. 6C shows the UV spectrum of QA-18. FIG. 6D shows the UV spectrum of QA-21.

FIG. 7A shows ^{1}H Nuclear Magnetic Resonance ("NMR") of QA-7. FIG. 7B shows ^{1}H NMR of QA-18. FIG. 7C shows ^{1}H NMR of QA-21.

FIG. 8A shows the mass spectroscopy-fast atom bombardment ("MS-FAB") spectrum of QA-7. FIG. 8B shows the MS-FAB spectrum of QA-17. FIG. 8C shows the MS-FAB spectrum of QA-21.

FIG. 9 shows the elution profile of pure QA-18 micelles and pure QA-21 micelles by gel filtration on Bio-Gel P-200 in PBS equilibrated with the critical micellar concentration of the same saponin and a comparison with the elution position of standard proteins.

FIG. 10 shows the hemolysis of sheep red blood cells by QA-7, QA-8, QA-17, QA-18, QA-21, and Superfos "Quil-A."

FIG. 11 shows the typical endpoint titers for immunization with BSA antigen in the presence of HPLC-purified fractions of bark extract. Absorbance due to antigen-specific antibody binding was plotted as a function of the logarithm of the sera dilution.

FIG. 12 demonstrates the comparison of the adjuvant effects of QA-7, QA-17, QA-18 and QA-21 at various antigen concentrations and with Freund's complete adjuvant on immunization with the antigen BSA.

FIG. 13 shows the adjuvant effects of HPLC-purified adjuvants used in conjunction with $Al(OH)_3$, another adjuvant, on the immunization with the antigen gp70R-delta.

FIG. 14 summarizes the effects of HPLC-purified Quillaja saponins alone and in combination with each

other and with another adjuvant on the immunization with the antigen alkylated gp70R-delta.

FIG. 15 shows a comparison of the adjuvant effects of QA-18, QA-18H, QA-21, and QA-21H on immunization with the antigen BSA.

SUMMARY OF THE INVENTION

A need exists for a substantially pure saponin that can be used as an adjuvant in relatively low quantities with low toxicity and side effects. Accordingly, the present invention provides substantially pure saponin adjuvants, the method for the purification thereof and a method for the use of the substantially pure saponins as immune adjuvants. The invention further includes immune response-provoking compositions comprising the saponin adjuvants in combination with an antigen component.

Adjuvant saponins have been identified and purified from an aqueous extract of the bark of the South American tree, *Quillaja saponaria Molina*. At least 22 peaks with saponin activity were separable. The predominant purified Quillaja saponins have been identified as QA-7, QA-17, QA-18, and QA-21. These saponins have been purified by high pressure liquid chromatography (HPLC) and low pressure silica chromatography. These four saponins have adjuvant effect in mice. QA-7, QA-17, QA-18, and QA-21, purified from Superfos "Quil-A," a crude Quillaja saponin preparation, are less toxic in mice than "Quil-A"; QA-17 and QA-18 are less toxic in cats than "Quil-A" (QA-7, QA-21 were not tested). In addition, a toxic component of Superfos "Quil-A" has been identified as QA-19; this component is toxic in mice at lower doses than "Quil-A" or QA-7, QA-17, QA-18, and QA-21. The increased toxicity of QA-19 compared to QA-7, QA-17, QA-18, and QA-21 is unexpected in that this component is a saponin, has a similar carbohydrate composition, exhibits adjuvant activity in mice at doses lower than the toxic dose, and exhibits similar chromatographic behavior. All of the above saponins may be isolated from aqueous extracts of *Quillaja saponaria Molina* bark. The substantially pure saponins of the present invention are useful as immune adjuvants and enhance immune responses in individuals at a much lower concentration than the previously available heterogeneous saponin preparations without the toxic effects associated with crude saponin preparations.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The saponins of the present invention may be obtained from the tree *Quillaja saponaria Molina*.

The term "saponin" as used herein includes glycosidic triterpenoid compounds which produce foam in aqueous solution, have hemolytic activity in most cases, and possess immune adjuvant activity. The invention encompasses the saponin per se, as well as natural and pharmaceutically acceptable salts and pharmaceutically acceptable derivatives. The term "saponin" also encompasses biologically active fragments thereof.

The invention also concerns compositions, such as immunologic compositions, comprising one or more substantially pure saponin fractions, and methods of using these compositions as immune adjuvants.

The term "immune adjuvant," as used herein, refers to compounds which, when administered to an individual or tested in vitro, increase the immune response to an antigen in the individual or test system to which said antigen is administered. Some antigens are weakly immunogenic when administered alone or are toxic to the individual at concentrations which evoke immune responses in said individual. An immune adjuvant may enhance the immune response of the individual to the antigen by making the antigen more strongly immunogenic. The adjuvant effect may also lower the dose of said antigen necessary to achieve an immune response in said individual.

The adjuvant activity of the saponins may be determined by any of a number of methods known to those of ordinary skill in the art. The increase in titer of antibody against specific antigen upon administration of an adjuvant may be used as a criteria for adjuvant activity (Dalsgaard, K. (1978) *Acta Veterinia Scandinavica* 69, 1–40, Scott, M. T., Gross-Samson, M., and Bomford, R. (1985) *Int. Archs. Allergy Appl. Immun.* 77, 409–412). Briefly, one such test involves injecting CD-1 mice intradermally with an antigen (for instance, i.e., bovine serum albumin, BSA) mixed with varying amounts of the potential adjuvant. Sera was harvested from the mice two weeks later and tested by ELISA for anti-BSA antibody. A comparison of the adjuvant effects of the dialyzed, methanolsoluble bark extract and "Quil A" showed that antibody titers were two orders of magnitude greater when the antigen BSA was administered in the presence of the saponin preparations than when BSA was administered in PBS alone. The bark extract possessed good adjuvant activity when administered at an adjuvant dose of 12 μg carbohydrate (assayed by anthrone) or more. The adjuvant response to "Quil-A" was lower than for the bark extract but was evident at doses ranging from 9–23 μg carbohydrate. Carbohydrate weight (determined by assay with anthrone using glucose as a standard) is approximately 30% of the dry weight of these crude adjuvant extracts.

The term "substantially pure" means substantially free from compounds normally associated with the saponin in its natural state and exhibiting constant and reproducible chromatographic response, elution profiles, and biologic activity. The term "substantially pure" is not meant to exclude artificial or synthetic mixtures of the saponin with other compounds.

Preferably, the substantially pure saponin is purified to one or more of the following standards: 1) appearing as only one major carbohydrate staining band on silica gel TLC (EM Science HPTLC Si60) in a solvent system of 40 mM acetic acid in chloroform/methanol/water (60/45/10, v/v/v), 2) appearing as only one major carbohydrate staining band on reverse phase TLC (EM Science Silica Gel RP-8) in a solvent system of methanol/water (70/30, v/v), 3) appearing as only one major peak upon reverse-phase HPLC on Vydac C4 (5 μm particle size, 330 Å pore, 4.6 mm ID × 25 cm L) in 40 mM acetic acid in methanol/water (58/42, v/v).

In the preferred embodiment, the saponin adjuvants of the present invention are purified from *Quillaja saponaria Molina* bark. Aqueous extracts of the *Quillaja saponaria Molina* bark were dialyzed against water. The dialyzed extract was lyophilized to dryness, extracted with methanol and the methanol-soluble extract was further fractionated on silica gel chromatography and by reverse phase high pressure liquid chromatography (RP-HPLC). The individual saponins were separated by reverse phase HPLC as described in Example 1. At least 22 peaks (denominated QA-1 to QA-22) were separable. Each peak corresponded to a carbohydrate peak as demonstrated in FIG. 2 and exhibited only a single band on reverse phase thin layer chromatography. The

individual components were identified by retention time on a Vydac C_4 HPLC column as follows:

Peak	Retention Time (minutes)
QA-1	solvent front
QA-2	4.6
QA-3	5.6
QA-4	6.4
QA-5	7.2
QA-6	9.2
QA-7	9.6
QA-8	10.6
QA-9	13.0
QA-10	17.2
QA-11	19.0
QA-12	21.2
QA-13	22.6
QA-14	24.0
QA-15	25.6
QA-16	28.6
QA-17	35.2
QA-18	38.2
QA-19	43.6
QA-20	47.6
QA-21	51.6
QA-22	61.0

Immune adjuvant activity was tested by measuring the ability of the purified saponins to enhance the immune response in mice to exogenously administered antigens. The purified saponins of the present invention demonstrated adjuvant effects at lower doses than the crude extracts. Particularly, the predominant saponins in bark extract (QA-7, QA-17, QA-18, and QA-21) demonstrated adjuvant activity at doses of 4.5 μg carbohydrate or less (assayed by anthrone). The purified saponins were further characterized by carbohydrate content, reverse phase and normal phase TLC, UV, infra red, NMR spectra, and fast atom bombardment—mass spectroscopy.

The approximate extinction coefficient determined for 1% (w/v) solutions in methanol at 205 nm of several of the more preferred purified saponins are as follows:

	1% E_{205} (nm)
QA-7	34
QA-17	27
QA-18	27
QA-21	28

Carbohydrate content was used to quantitate the saponins in some instances. The carbohydrate assay was the anthrone method of Scott and Melvin (*Anal. Chem.* 25:1656 (1953)) using glucose as a standard as described in Example 1. This assay was used to determine a ratio of extent of anthrone reaction (expressed in glucose equivalents) per mg of purified saponin (dry weight) so that dry weight of a particular preparation could be estimated by use of anthrone assay. It must be noted that differences in reactivity with anthrone for different saponins may be due to carbohydrate composition rather than quantity as different monosaccharides react variably in this assay.

The substantially pure QA-7 saponin is characterized as having immune adjuvant activity, containing about 35% carbohydrate (as assayed by anthrone) per dry weight, having a uv absorption maxima of 205–210 nm, a retention time of approximately 9–10 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID × 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 52–53% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mM ID × 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of approximately 0.06% in water and 0.07% in phosphate buffered saline, causing no detectable hemolysis of sheep red blood cells at concentrations of 200 μg/ml or less, and containing the monosaccharide residues terminal rhamnose, terminal xylose, terminal glucose, terminal galactose, 3-xylose, 3,4-rhamnose, 2,3-fucose, and 2,3-glucuronic acid, and apiose (linkage not determined).

The substantially pure QA-17 saponin is characterized as having adjuvant activity, containing about 29% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205–210 nm, a retention time of approximately 35 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID × 25 cm L in a solvent of 40 mM acetic acid in methanol-water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 63–64% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mm ID × 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of 0.06% (w/v) in water and 0.03% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood cells at 25 μg/ml or greater, and containing the monosaccharide residues terminal rhamnose, terminal xylose, 2-fucose, 3-xylose, 3,4-rhamnose, 2,3-glucuronic acid, terminal glucose, 2-arabinose, terminal galactose and apiose (linkage not determined).

The substantially pure QA-18 saponin is characterized as having immune adjuvant activity, containing about 25–26% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205–210 nm, a retention time of approximately 38 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID × 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 64–65% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mm ID × 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, having a critical micellar concentration of 0.04% (w/v) in water and 0.02% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood cells at concentrations of 25 μg/ml or greater, and containing the monosaccharides terminal rhamnose, terminal arabinose, terminal apiose, terminal xylose, terminal glucose, terminal galactose, 2-fucose, 3-xylose, 3,4-rhamnose, and 2,3-glucuronic acid.

The substantially pure QA-21 saponin is characterized as having immune adjuvant activity, containing about 22% carbohydrate (as assayed by anthrone) per dry weight, having a UV absorption maxima of 205–210 nm, a retention time of approximately 51 minutes on RP-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID × 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/min, eluting with 69 to 70% methanol from a Vydac C_4 column having 5 μm particle size, 330 Å pore, 10 mm × ID 25 cm L in a solvent of 40 mM acetic acid with gradient elution from 50 to 80% methanol, with a critical micellar concentration of about 0.03% (w/v) in water and 0.02% (w/v) in phosphate buffered saline, causing hemolysis of sheep red blood

cells at concentrations of 25 μg/ml or greater, and containing the monosaccharides terminal rhamnose, terminal arabinose, terminal apiose, terminal xylose, 4-rhamnose, terminal glucose, terminal galactose, 2-fucose, 3-xylose, 3,4-rhamnose, and 2,3-glucuronic acid.

The term "individual" means any animal which can elicit an immune response, including humans.

The purified saponins exhibit adjuvant effects when administered over a wide range of dosages and a wide range of ratios to the antigen being administered. In one embodiment, the saponin is administered in a ratio of adjuvant to antigen (w/w) of 3.0 or less, preferably 1.0 or less.

The purified saponins may be administered either individually or admixed with other substantially pure adjuvants to achieve the enhancement of the immune response to an antigen. Among the adjuvant mixtures effective in the present invention are fractions QA-7 and QA-17, QA-7 and QA-18, QA-17 and QA-18, or QA-7, QA-17, and QA-18 administered together. Purified saponins may also be administered together with non-saponin adjuvants. Such non-saponin adjuvants useful with the present invention are oil adjuvants (for example, Freund's Complete and Incomplete), liposomes, mineral salts (for example, $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)$, silica, alum, $Al(OH)_3$, $Ca_3(PO_4)_2$, kaolin, and carbon), polynucleotides (for example, poly IC and poly AU acids), and certain natural substances (for example, wax D from *Mycobacterium tuberculosis*, as well as substances found in *Corynebacterium parvum*, *Bordetella pertussis*, and members of the genus Brucella).

The purified saponins of the present invention may be utilized to enhance the immune response to any antigen. Typical antigens suitable for the immune-response provoking compositions of the present invention include antigens derived from any of the following: viruses, such as influenza, rabies, measles, hepatitis B, hoof and mouth disease, or HTLV-III; bacteria, such as anthrax, diphtheria or tuberculosis; or protozoans, such as *Babeasis bovis* or Plasmodium.

A particular example is the use of the purified saponins of the present invention to enhance the immune response to gp70 recombinant protein. One gp70 recombinant protein is an antigen which contains the polypeptide portion of FeLV gp70 envelope protein. This recombinant antigen is termed "gp70R," "rec-gp70" or "Rgp70." Another antigen preparation which contains the polypeptide portion of FeLV gp70 together with the 40 amino-terminal amino acids (termed "Rgp70delta") or with the entire amino acid sequence (termed "Rgp90") of the p15e envelope protein of FeLV subgroup A is produced using recombinant DNA techniques. These recombinant gp70-containing polypeptides, gp70R, gp70R-delta, and gp90R, are hereinafter referred to collectively as gp70-containing protein. The term gp70-containing protein is intended to include polypeptides having the same amino acid sequence of the naturally occurring gp70-containing protein, and analogs thereof. The term "analogs" is intended to include proteins or polypeptides which differ from gp70, gp70-delta, or gp90 by addition, deletion or substitution of one or more amino acids providing that said polypeptide demonstrate substantially the biological activity of gp70 protein.

Administration of the compounds useful in the method of present invention may be by parenteral, intravenous, intramuscular, subcutaneous, intranasal, or any other suitable means. The dosage administered may be dependent upon the age, weight, kind of concurrent treatment, if any, and nature of the antigen administered. The effective compound useful in the method of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert carrier is preferably used, such as saline, or phosphate-buffered saline, or any such carrier in which the compounds used in the method of the present invention have suitable solubility properties for use in the method of the present invention.

Having now generally described the invention, the same may be further understood by reference to the following examples, which are not intended to be limiting unless so expressly stated.

EXAMPLE 1

Preliminary Preparation of *Quillaja Saponaria Molina* Bark Extract

Quillaja saponaria Molina bark was stirred with an excess of water (10% w/v) to extract the saponins. The aqueous extract was then filtered and stored in 0.1% NaN_3. 150 ml of this extract was centrifuged at 20,000×g for 30 minutes to remove residual bark fragments. The supernatant, which was light brown, was lyophilized and redissolved in 16 ml of water and the pH was adjusted to less than 4 with the addition of 160 μl of 1N acetic acid. This solution was placed in dialysis tubing having a 12,000 MW cut off and dialyzed against 1 liter of water. The water was changed after 8 hours of dialysis, and the dialysis was allowed to proceed overnight. Samples of the dialysate were removed after the first and second dialysis cycles. The dialyzed extract was lyophilized and extracted with 40 ml methanol at 60° C. for 15 minutes followed by centrifugation at 1,000×g for 10 minutes to sediment the undissolved material. This material was subjected to two additional extractions with methanol. The methanol extracts were pooled, evaporated on a rotoevaporator to dryness, redissolved in 5.5 ml methanol, and filtered through a 0.2μ nylon 66 mesh to remove residual undissolved material. Fractions were analyzed by reverse phase thin-layer chromatography (RP-TLC) on C8 plates (E.M. Science RP-TLC, C8) in a solvent system of 70% methanol/30% water or by normal phase thin layer chromatography on silica gel 60 TLC plates in a solvent system of n-butanol, ethanol, water, and ammonia (30/60/29/21, v/v/v/v). The carbohydrate bands were visualized with Bial's reagent which detected all major bands detectable by sulfuric acid charring with an increased sensitivity over the sulfuric acid charring method. The Bial's reagent carbohydrate stain was routinely used as a detection reagent on TLC plates. All major bands were glycosylated.

Dialysis removed a major carbohydrate-containing band (R_f=0.82 on EM Science RP TLC, C8 in methanol/water (70/30, v/v)), as well as some minor components. In addition, dialysis removed components with strong absorption maxima at 280 and 310 nm. Approximately 80% of the carbohydrate (assayed by anthrone) was removed by dialysis, but about 95% of the hemolytic activity was retained during dialysis.

Most saponin adjuvants are known to have detergent properties, such as hemolysis of red blood cells, so the retention of hemolytic activity is a rough indication of the retention of adjuvant saponins. Several bands were retained by dialysis, indicating their detergent nature.

Methanol solubilized all TLC bands present in the dialyzed extract except one TLC band (R_f=0 on both reverse-phase and silica TLC plates). The methanol-insoluble material was reddish-brown. The material which was methanol-soluble appeared white after lyophilization.

Carbohydrate concentration was determined by the method of Scott and Melvin (Scott, T. A., and Melvin, E. H. *Anal. Chem.* 25, 1656 (1953)). Briefly, an aqueous sample to be tested or glucose as a standard carbohydrate solution (450 μl) was mixed with 900 μl of 0.2% anthrone (w/v) in sulfuric acid and incubated for 16 min at 90°-100° C. The absorbance was read at 625 nm. Glucose was used as a standard.

The hemolytic activity of the samples was determined as follows: Briefly, samples were diluted in a round bottom microtiter plate with 1:2 dilutions in phosphate buffered saline in successive rows (100 μl/well). 10 μl normal rabbit blood in Alsevers solution (Hazelton) was added to each well and mixed. Plates were incubated for one hour at room temperature followed by centrifugation of the plates in a Sorvall RT6000 to sediment unhemolyzed cells. Absence of hemolysis was determined by the presence of a pellet of unhemolyzed cells in the bottom of the well.

EXAMPLE 2

Comparison of Dialyzed, Methanol-Soluble Bark Extract and Superfos "Quil-A" by TLC and HPLC

Superfos "Quil-A" and dialyzed, methanolsoluble components of bark extract prepared as in Example 1 were compared by reverse phase TLC as described in Example 1. All bands present in the bark extract after dialysis and solubilization with methanol were present in "Quil-A." In addition, "Quil-A" contained a band with r_f=0 on reverse-phase TLC plates; this component was removed by methanol-solubilization as described above. The similarity in composition of dialyzed, methanolsoluble bark extract and "Quil-A" was confirmed by HPLC. The individual components of bark extract were separable by reverse-phase HPLC on Vydac C4 (5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L) in 40 mM acetic acid in methanol/water (58/42, v/v). The refractive index of the individual fractions was determined. FIG. 1 represents the refractive index profile of the peaks (labeled QA-1 to QA-22 in order of increasing retention times) from the RP-HPLC. The relative proportion of each peak in bark extract and Superfos "Quil-A" is shown on Table 1, below.

TABLE 1

	Relative proportion of HPLC fractions of crude saponin extract and Superfos "Quil-A" (refractive index) % of Total (peaks 2-21)	
HPLC Fraction	Dialyzed, methanol-soluble bark extract	Superfos "Quil-A"
QA-2	3.1	1.2
QA-3	4.8	2.4
QA-4,5	10.1	7.1
QA-6,7	17.3	12.7
QA-8	6.8	10.5
QA-9	1.0	2.1
QA-10	2.7	1.3
QA-11	6.8	6.2
QA-12	3.5	5.6
QA-13,14,15	4.8	7.7
QA-16	2.8	1.4
QA-17	11.4	9.9

TABLE 1-continued

	Relative proportion of HPLC fractions of crude saponin extract and Superfos "Quil-A" (refractive index) % of Total (peaks 2-21)	
HPLC Fraction	Dialyzed, methanol-soluble bark extract	Superfos "Quil-A"
QA-18	13.5	21.8
QA-19	2.2	4.5
QA-20	3.2	2.2
QA-21	5.6	3.7

The individual peaks correspond to single thin-layer chromatography bands on reverse-phase TLC plates. Another representative experiment shown on FIG. 2 demonstrates that the refractive index peaks also correspond to carbohydrate peaks, confirming that all major bark extract components are glycosides (HPLC fractions assayed for carbohydrate by the anthrone assay).

Dialyzed, methanol-soluble bark extract and "Quil-A" were compared directly in this HPLC system. The individual components were identified by retention time. All peaks present in dialyzed, methanol-soluble bark extract were also present in "Quil-A" in similar proportions with the exception of a higher proportion of component QA-8 and a lower proportion of component QA-17 in Superfos "Quil-A" compared to bark extract. FIG. 3 shows a comparison of dialyzed, methanol-soluble bark extract and Superfos "Quil-A" using a semipreparative Vydac C_4 (10 mm ID×25 cm L, 330 Å pore size, 5 μm particle size). The sample is loaded in 50% methanol in 40 nM acetic acid and a methanol gradient in 40 mM acetic acid (shown in FIG. 3) is used to elute the samples. The absorbance was monitored at 214 nm.

Various samples of Quillaja bark were extracted and analyzed by HPLC. There was some variability in the relative proportions of the peaks, but the same peaks were always present. It is not presently known whether the variability in proportions is due to variability in the efficiency of the extraction process or in bark from different sources.

Due to the ready availability of "Quil-A" and the similar composition to bark extract, "Quil-A" was utilized to produce mg quantities of material. Adjuvant activity in mice, using BSA as antigen, was found to be associated with peaks 4, 7, 11, 12, 15, 16, 17, 18, 19, and 20 (Table 2) at doses of 3.0 μg carbohydrate (determined by the anthrone assay). The absorbance due to antigen-specific antibody binding (two weeks post-immunization, determined by ELISA) at a sera dilution of 1:10 provides a semi-quantitative estimate of adjuvant activity (ranging from 0.07 in mice immunized in the absence of adjuvant to 1.24 in mice immunized in the presence of QA-20).

TABLE 2

	Adjuvant Activity in Mice	
HPLC Fraction	Adjuvant Dose (μg carbohydrate)	Absorbance* (410 nm)
QA-2	3.0	.34
QA-3	3.0	.27
QA-4	3.0	.60
QA-7	3.0	.49
QA-10	3.0	.13
QA-11	3.0	.46
QA-12	3.0	.76
QA-13,14	3.0	.20
QA-15	3.0	1.17

TABLE 2-continued

HPLC Fraction	Adjuvant Activity in Mice: Adjuvant Dose (μg carbohydrate)	Absorbance* (410 nm)
QA-16	3.0	.66
QA-17	3.0	1.13
QA-18	3.0	.75
QA-19	3.0	.93
QA-20	3.0	1.24
		0.07

*Absorbance due to antigen-specific antibody binding at sera dilution of 1:10.

Due to the predominance of peaks QA-7, QA-17, QA-18, and QA-21 in bark extract, these four components were purified on a layer scale, as described in Examples 3 and 4, below.

EXAMPLE 3

Purification by Silica Chromatograph

1 gram "Quil-A" was suspended in 75 ml methanol and heated at 60° for 15 minutes and filtered. The undissolved material was extracted a second time with 50 ml methanol at 60° C. and filtered. The filtrates were evaporated to dryness on the rotoevaporator. A Lichropep Silica Si60 column (E.M. Science, 25 mm ID × 310 mm L, 40–63 μm particle size) was pre-equilibrated in 40 mM acetic acid in chloroform/methanol/water (62/32/6, v/v/v).

The dried "Quil-A," a crude mixture of saponins, was dissolved in 5 ml of column solvent and eluted through the silica isocratically in this solvent system at a flow rate of 1 ml/min. Carbohydrate analysis, thin-layer chromatography, and HPLC were used to monitor the fractions for QA-7, QA-17, QA-18, and QA-21. Fractions 19–30 were enriched in QA-21 and were pooled for further purification of QA-21. Fractions 31–60 were enriched in QA-8 and QA-18 and were pooled for further purification of these components. Fractions 85–104 were enriched with QA-7 and QA-17 and were pooled for further purification of these components. These pools were flash evaporated prior to further purification.

EXAMPLE 4

Further Purification by Reverse Phase HPLC

Silica fractions were further purified by semipreparative reverse phase HPLC on Vydac C_4 (10 mm ID × 25 cm L), FIG. 4. Silica fractions (10–20 mg) were dissolved in the appropriate solvent and loaded on Vydac C_4. A methanol gradient was used to elute the fractions. The flow rate was 3 ml per minute. The fractions were monitored by absorbance at 214 nm. FIG. 4B shows the purification of QA-21 from silica fractions 19–30 using isocratic separation in 40 mM acetic acid in 58% methanol/42% water. Fractions eluting with a retention time between 65–72 minutes were identified as QA-21 by reverse phase TLC and pooled for further characterization. FIG. 4C shows the purification of QA-18 from silica fractions 31–60 using a methanol gradient in 40 mM acetic acid (50–56% methanol/0–10 min, 56–69% methanol/10–79 min). Fractions eluting with a retention time between 46–48 minutes were identified as QA-18 by reverse phase TLC and pooled for further characterization. FIG. 4D shows the purification of QA-7 and QA-17 from silica fractions 85–104 using the same gradient used in FIG. 4C. Fractions eluting with a retention time between 21–23 minutes were identified as QA-17 by reverse phase TLC and pooled for further characterization. Fractions eluting with a retention time between 44–46 minutes were identified as QA-17 by reverse phase TLC and pooled for further characterization.

EXAMPLE 5

Purity and Characterization of Adjuvants Purified by Silica and Reverse Phase Chromatography

Purity

FIG. 5*a* represents a reverse-phase TLC (E.M. Science RP-TLC, C8 (Solvent = 70% methanol, visualization spray = Bial's reagent)). 5 μg each of QA-7, QA-17, QA-18, and QA-21 purified as described in Example 3 and 4, were chromatographed. The adjuvants each appeared as single bands in this TLC system.

FIG. 5*b* demonstrates fractions QA-7, QA-17, QA-18, QA-21 and "Quil-A" on EM Si60 HPTLC plate (solvent = 40 mM acetic acid in chloroform/methanol/H_2O (60/45/10, v/v/v), visualization spray = Bial's reagent). 2 μg each of QA-7, QA-17, QA-18 and QA-21, purified as described in Examples 3 and 4, and 20 μg of "Quil-A," a crude saponin extract, were chromatographed. The HPLC-purified material appeared predominantly as a single band.

Spectroscopy

The UV spectra of QA-7, QA-17, QA-18 and QA-21 in methanol are shown on FIGS. 6A–D respectively. Dalsgaard's (Dalsgaard, K., *Acta Veterinaria Scandinavica Supp.* 69:1–40 (1978)) adjuvant fraction had an absorbance peak at 280 nm; however, the HPLC-purified fractions of the present invention do not have a peak at 280 nm but have a major peak in the region between 200–220 nm with a shoulder centered at 260 nm.

Fourier Transform-Infrared Resonance ("FT-IR") spectra showed little difference between the adjuvants, suggesting that they all have the same functional groups. Although identification of the structure cannot be made from the IR, the spectral data is consistent with the presence of a carboxyl group as was suggested by Dalsgaard (Dalsgaard, K., supra).

^{1}H-NMR at 250 MHz of the purified saponins in CD_3OD demonstrates the complex nature of the purified saponins QA-7 (FIG. 7A), QA-18 (FIG. 7B), and QA-21 (FIG. 7C). The signals in the region between 4.1 to 5.4 ppm clearly demonstrate the presence of multiple signals from the anomeric protons of the monosaccharides, indicating a multiplicity of monosaccharide residues. However, the NMR spectra of the saponins are too complex to allow structural determination.

MS-FAB of the purified saponins QA-7, QA-17, and QA-21 (FIGS. 8A, 8B, 8C, respectively) indicated approximate pseudo- molecular ion masses of 1870, 2310, and 1980, respectively. MS-FAB was not determined on QA-18 due to difficulties in solubilizing this component. These molecular weights are consistent with those expected for a triterpene linked to eight to ten monosaccharide residues and were in the same range as monomer molecular weights determined by size exclusion HPLC of purified saponins in methanol (Zorbax PSM 60 Si column, 25 cm × 6.2 mm, 1 ml/min flow rate, molecular weight standards = 18-β-glycrhetinic acid and ginenoside Rb_1) which indicated approximate molecular weights of 2600, 2400, 1800, and 2400 for QA-7, QA-17, QA-18, and QA-21, respectively. The difference between FAB-MS and size exclusion HPLC are

most likely due to variation in shape between the saponins and the molecular weight standards.

Carbohydrate Composition

Table 3 below shows the carbohydrate composition and linkage analysis of purified saponins QA-7, QA-17, QA-18, QA-21, and QA-19. The carbohydrate in saponins was converted to alditol acetates by heating 0.2 mg saponin in 0.3 ml 2N trifluoroacetic acid containing 0.1 mg/ml inositol at 120° C. for two hours. The acid was removed under a flow of air, and residual acid removed by the addition of isopropanol (2×0.25 ml), followed by blowing to dryness with air. The dry residue obtained was dissolved in 1M ammonium hydroxide (0.25 ml) containing 10 mg/ml sodium borodeuteride and kept for one hour at room temperature. Glacial acetic acid (0.1 ml) was added, and the solution was blown to dryness. Residual borate was removed by co-distilling with 10% acetic acid in methanol (3×0.25 ml) and finally with methanol (2×0.25 ml). The dry residue in acetic anhydride (0.1 ml) and pyridine (0.1 ml) was heated for 20 minutes at 120° C. Toluene (9.02 ml) was added to the cooled solution, and the solvents removed under a flow of air. This procedure of adding toluene and removing pyridine and acetic anhydride was repeated twice. The residue obtained was taken up in dichloromethane (0.5 ml) and extracted with water (0.5 ml). The organic phase was transferred to a clean tube and dried. Prior to analysis by GLC (gas-liquid chromatography), the residue was dissolved in acetone (0.1 ml). Alditol acetates were analyzed on an SP2330 capillary GLC column (30 m×0.25 mm) at 235° C.) with flame ionization detection. The carbohydrate in saponins was converted to trimethylsilated methylglycosides by heating 0.1 mg of sample in methanolic HCl (0.3 ml) containing 50 ug/ml inositol for 16 hours at 80° C. The sample was blown to dryness, and residual acid removed by the addition of t-butyl alcohol (2×0.25 ml) followed by drying with a flow of air. The dry residue was dissolved in a solution (0.2 ml) containing pyridine, hexamethyldisilazane, and trimethylchlorosilane (5:1:0.5 v/v, "Tri-Sil") and heated for 20 minutes at 80° C. The silylating reagent was evaporated at room temperature, and the residue dissolved in hexane (1 ml). After removal of the insoluble residue by filtration using glass wool plug, the filtrate was transferred to a clean tube and evaporated. The residue was dissolved in hexane (0.2 ml) prior to analysis by GLC. The trimethylsilated methyl glycosides were analyzed on a GLC column of fused silica DB1 (25 m×0.25 mm) for 3 min at 160° C. followed by a 2°/min increase to 200° C. and then a 10°/min increase to 260° C. with flame ionization detection.

Glycoside linkage analysis was carried out by the following method: To the sample (≃1 mg) dissolved in dry dimethylsulfoxide (0.2 ml), 0.2 ml of potassium dimethylsulphinyl anion (2M) was added, and the mixture stirred for 12 hours under argon. The reaction mixture was cooled in ice, and methyl iodide (0.2 ml) was added drop wise. The resulting mixture was sonicated and stirred at room temperature for one hour. The methylated material was isolated using Sep-Pak C_{18} cartridges conditioned with ethanol (20 ml), acetonitrile (8 ml), and water (10 ml). Water (1 ml) was added to the methylation reaction mixture, and the excess methyl iodide removed by passing nitrogen through the solution. The clear solution was applied to the cartridge which was washed with water (8 ml) and 20% acetonitrile (5 ml). The methylated material was eluted from the cartridge with 100% acetonitrile (4 ml) and ethanol (4 ml). The solvents were removed with a flow of air. The dried methylated material was treated with 0.3 ml of "super deuteride" solution at room temperature for one hour in order to reduce the uronic acid residues to the corresponding hexoses. After destroying the excess reagent with glacial acetic acid (0.1 ml), the reaction mixture was blown to dryness with 10% acetic acid/-methanol and blown to dryness two more times. The resulting reduced methylated material in methanol was passed through a column of Dowex—50 W(H+) and the effluent obtained was dried. The reduced methylated material was converted to methylated alditols as described in section 1 above and analyzed by GLC (SP2330 fused silica column (30 m×0.25 mm), 3 min at 170° C. followed by 4°/min to 240° C.) and GLC-MS (SP2330 fused silica column (30 m×0.25 mm), 2 min at 80° C. followed by 30°/min to 170° C. followed by 4°/min to 240° C. followed by holding at 240° C. for 10 min, mass spectral analysis on Hewlett-Packard MSD).

Despite the similarity in the carbohydrate composition, subtle differences distinguish the individual saponins, in particular, the absence of arabinose in QA-7 and decreased glucose in QA-21 compared to the other saponins.

TABLE 3

Carbohydrate Composition and Linkage Analysis of Purified Saponins

	QA-7			QA-17			QA-18			QA-19A			QA-21		
	AA[a]	TMS[b]	Linkage	AA	TMS	Linkage	AA	TMS	Linkage	AA	TMS	Linkage	AA	TMS	Linkage
rhamnose	191.4	1.57	T[c]	184.8	1.9	T	132.0	0.99	T	32.7	1.69	T	131.9	1.07	T
			3,4			3,4			3,4			3,4			4
fucose	86.7	0.67	2,3	77.9	0.78	2	95.6	0.76	2	26.6	0.88	2	99.8	0.76	2
arabinose	trace	trace		65.4	0.80	2	80.1	0.64	T	31.1	0.94	T	71.0	0.65	T
xylose	98.1	0.95	T	81.8	1.08	T	117.8	1.16	T	49.9	2.07	T	114.3	1.21	T
			3			3			3			3			3
galactose	81.2	0.74	T	69.4	0.81	T	88.1	0.86	T	trace	1.11	T	88.1	0.84	T
glucose	81.2	1.0	T	85.7	1.0	T	89.2	1.00	T	trace	1.0	T	19.6	0.30	T
glucuronic acid	N.T.[d]	0.48	2,3	N.T.	0.52	2,3	N.T.	0.62	2,3	29.2	0.62	2,3	N.T.	0.62	2,3
apiose[e]	22.5	N.T.		24.5	N.T.		25.7	N.T.	T	27.7		T	20.0	N.T.	T

[a]Alditol acetate (μg/mg saponin)
[b]Trimethylsilated methyl glycosides (relative proportions)
[c]T-terminal glycosyl residue, that is, attached through C-1 but with no other residues attached to it. 3,4 = a glycosyl residue attached through C-1 with other glycosyl residues glycosidically attached to it through C-3 and C-4.
[d]Not tested
[e]Poor recovery as alditol acetates

Characterization of Saponins as Detergents

The critical micellar concentration of adjuvants QA-7, QA-17, QA-18, and QA-21 was determined by the method of DeVendittis et al. (DeVendittis, E., Palumbo, G., Parlato, G., and Bocchini, V. (1981) *Anal.*

Biochem. 115, 278-286) as follows: The emission spectrum of 1-anilinonapthalene-8-sulfonic acid (ANS) in water was determined at dry weight concentrations of adjuvant ranging from 0.01 to 0.10% (W/v) to cover the range below and above the critical micellar concentration. Above the critical micellar concentration, the fluorescence yield of ANS increases and the wavelength of maximum emission decreases due to partitioning of the fluorescent dye into the micelles. Similar critical micellar concentrations were found for QA-7, QA-17, QA-18, and QA-21 in water (0.06%, 0.06%, 0.04%, and 0.03%, respectively) with slightly lower concentrations determined in phosphate buffered saline (0.07% 0.03%, 0.02%, and 0.02%, respectively).

FIG. 9 shows the gel filtration chromatograph for micelles formed by purified QA-18 and QA-21 (on Bio-Gel P-200 (6.6 mm ID×90 cm ht)), pre-equilibrated in a concentration of purified saponin equivalent to the critical micellar concentration of that saponin in phosphate buffer saline to prevent the monomer-micelle equilibrium from reducing the apparent radius of the micelles). QA-18 and QA-21 micelles elute with a size that is similar to that of the protein bovine serum albumin.

The hemolytic activity of the adjuvants was determined by the following method: Dilutions of adjuvants QA-7, QA-8, QA-17, QA-18, QA-21, and Superfos "Quil-A" were made on a round bottom , microtiter plate (75 μl per well). Sheep red blood cells (SRBC), washed three times with PBS, were diluted to 4% with PBS. SRBC (25 μl) were added to each well and mixed with adjuvant. After incubation at room temperature 30 min, the plates were spun at 1000 rpm 5 min in a Sorvall RT6000, H-1000 rotor, to sediment unhemolyzed cells. 50 μl of the supernatant from each well was transferred to the same well of a flat bottom microtiter plate and diluted to 200 μl with H_2O. Absorbance was determined at 570 nm with a Dynatech microtiter plate reader. (FIG. 9) Hemolysis increased the absorbance at 570 nm due to release of hemoglobin from the lysed cells. Significant differences in hemolysis were observed between adjuvants. QA-17, QA-18, QA-21, and Superfos "Quil-A" caused partial hemolysis at concentrations as low as 25 μg/ml whereas partial hemolysis was observed with QA-8 at 150 μg/ml. No hemolysis was observed with QA-7 at the concentrations tested (200 μg/ml and less).

EXAMPLE 6

Isolation of Toxic Component QA-19

The toxic component QA-19 cochromatographs with QA-18 on silica and is enriched in silica fractions 31-60. These fractions were pooled and flash evaporated prior to further purification. FIG. 4C shows the separation of QA-19 from QA-18 by reverse phase HPLC on Vydac C_4 (10 mm ID×25 cm L) using a methanol gradient. Fractions eluting with a retention time between 50-52 minutes were identified as QA-19 by reverse phase TLC and analytical HPLC and pooled for further characterization. QA-19 could be further separated into two peaks by repurification in a shallower methanol gradient, with the peak with shorter retention time designated QA-19a and the peak with longer retention time designated QA-19b. Carbohydrate analysis of peak QA-19a which is more toxic in mice than QA-19b, shows a carbohydrate composition which is similar to that of the other saponins (Table 3).

EXAMPLE 7

Isolation of Alkaline Hydrolysis Product

Treatment of QA-18 by brief alkaline hydrolysis yielded one major carbohydrate-containing alkaline hydrolysis product (designated QA-18 H). Purified QA-18 H was prepared from QA-18 and isolated in the following manner:

One ml QA-18 (5 mg/ml) was incubated with 25 μl 1N NaOH for 15 minutes at room temperature. The reaction was stopped with the addition of 100 μl 1N acetic acid. Using these hydrolysis conditions, QA-18 was completely converted to a major hydrolysis product (QA-18 H) eluting in a peak with retention time of 8.0 min compared to 66.8 min for unhydrolyzed QA-18, indicating the increased hydrophilicity of QA-18 H. (Chromatography on Vydac C_4 (4.6 mm ID×25 cm L) in 0.1% trifluoroacetic acid in 55/45 methanol/water v/v) and eluted in a gradient to 64/36 methanol/water (v/v) over 180 minutes, flow rate of 1 ml/minute). The peak containing pure QA-18 H (retention time 8.0 min) was pooled for further characterization. The hydrolysis product of QA-21, designated QA-21 H, was prepared and purified in the same manner. QA-21 H had a retention time of 9.3 minutes compared to 80.4 minutes for unhydrolyzed QA-21. These hydrolysis products were shown by retention time on HPLC and by reverse phase thin layer chromatography to be identical to the major hydrolysis products generated using the method of Higuchi et al., *Phytochemistry* 26: 229 (1987) using mild alkaline hydrolysis in NH_4HCO_3 (Table 4). In addition, these products, QA-18 H and QA-21 H, were shown to be the major breakdown products from hydrolysis of "Quil-A", a crude saponin mixture containing QA-7, QA-17, QA-18, and QA-21 as well as other saponins, indicating that the hydrolysis products QA-21 H and QA-18 H are the same hydrolysis products isolated by Higuchi et al., supra, for structural characterization. QA-18, H and QA-21 H were saved for further characterization of adjuvant activity.

TABLE 4

	Retention Time of Major Alkaline Hydrolysis Products
QA-17 H	8.0[a]
QA-18 H	8.0[a]
	8.2[b]
QA-21 H	9.3[a]
	9.5[b]
Hydrolyzed - "Quil-A"	8.2[a], 9.3[a]

[a]Cambridge BioScience hydrolysis conditions: 5 mg/ml saponin, pH 13, reaction time = 15 minutes at room temperature
[b]Higuchi et al. hydrolysis conditions: 5 mg/ml saponin, 6% NH_4HCO_3, methanol/-H_2O (1/1, v/v), reaction time = 60 minutes at 100° C.
HPLC Conditions:
Vydac C^4, 5 μm particle size, 300 Å pore size, .46 × 25 cm
Solvent A = 0.1% trifluoroacetic acid in water
Solvent B = 0.1% trifluoroacetic acid in methanol
Gradient = 55-64% B/180 minutes
Flow rate = 1 ml/min

EXAMPLE 8

Testing for Adjuvant Effect Using BSA as Antigen

Briefly, adjuvant effect is assessed by increase in antigen-specific antibody titers due to addition of potential adjuvant in the immunization formulation. Increased titers result from increased antibody concentrations and/or increased antigen/antibody affinity. Adjuvant effects of saponins have previously been measured by increase in titer of neutralizing antibodies to foot-and-

mouth disease vaccines in guinea pigs (Dalsgaard, K., *Archiv. fur die gesamte Virusforschung* 44, 243-254 (1974)), increase in titer of precipitating antibodies to BSA (as measured by radial immunodiffusion) in guinea pigs vaccinated with BSA/saponin mixtures (Dalsgaard, K. *Acta Veterinaria Scandinavica* 69, 1-40 (1978)), as well as by the increase in titer of anti-keyhole limpet hemocyanin (KLH) antibody (measured by ELISA) in mice immunized with KLH/saponin (Scott, M. T., Gross-Samson, and Bomford, R., *Int. Archs. Allergy Appl. Immun.* 77:409-412 (1985)).

Assessment of adjuvant effect in this study was determined by increase in anti-BSA antibody following immunization with BSA/saponin compared with immunization with BSA in the absence of saponin. The adjuvant activity in the purified fraction was measured as follows: CD-1 mice (8-10 weeks old) were immunized intradermally with the following formulation: 10 μg BSA (Sigma 7030, fatty acid free) and Quillaja adjuvant (at doses ranging from 1.5-45 μg carbohydrate as measured by anthrone) in 200 μl PBS. Sera was harvested two weeks post-immunization. Anti-BSA antibody was determined by ELISA: Immulon II plates were coated overnight at 4° C. with 100 μl fatty acid free BSA (10 μg/ml in PBS) in rows, A, C, E, and G. Plates were washed twice with PBS. Nonspecific binding was prevented by incubating for 1.5 h at 37° C. with 100 μl diluent (2% Casein acid hydrolysate (Oxoid, w/v) in PBS) per well in all wells. Plates were washed four times with 0.05% Tween 20 in distilled water. Sera at dilutions of 10, 10^2, 10^3, and 10^4 were incubated in rows A+B, C+D, E+F, and G+H, respectively (100 μl/well) for 1 h at room temperature. Plates were washed as described above. Boehringer-Mannheim horse radish peroxidase conjugate goat anti-mouse antibody (1/5000 in 5% BSA in diluent) was incubated for 30 min at room temperature (100 μl per well, all wells). Plates were washed as described above. The extent of peroxidase reaction was determined by reaction with 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonate (30 minute reaction at room temperature, absorbance measured at 410 nm) or with 3,3',5,5'-tetramethylbenzidine (10 min reaction at room temperature, absorbance measured at 450 nm). The contribution of nonspecific antibody binding to the total antibody binding was removed by subtraction of the absorbance of the antigen-negative well from the absorbance of the antigen-positive well for each sera dilution. The absorbance due to antigen-specific binding was plotted as a function of the logarithm of the sera dilution. (FIG. 11) Typical endpoint titers were typically at a sera dilution of 10 or less for immunization in the absence of adjuvant and were as high as 10^3 in the presence of saponin adjuvant. Dialyzed, methanol-soluble bark extract at an adjuvant dose of 12 μg carbohydrate or greater (carbohydrate assayed by anthrone) increased titers by 2 orders of magnitude compared to BSA in PBS. A good adjuvant effect was observed at doses of "Quil-A" between 9-23 μg carbohydrate.

EXAMPLE 9

Adjuvant Testing of HPLC-Purified Extract Components

By the criteria described in Example 8, peaks QA-7, QA-11, QA-12, QA-15, QA-16, QA-17, QA-18, QA-19, and QA-20 have varying degrees of adjuvant effect with QA-15, QA-17, QA-18, QA-19, and QA-20 being particularly effective at a dose of 3.0 μg carbohydrate in this particular experiment. Due to the small number of mice used per immunization (2) and the natural variation in immune response between individual mice, this experiment cannot be used to quantitatively assess the relative adjuvant effect of these peaks. However, it provides a qualitative assessment of the presence of adjuvant activity. It must also be noted that the absence of apparent effect with QA-2, QA-3, QA-10, QA-13, and QA-14 does not rule out an adjuvant effect at different adjuvant doses or adjuvant/protein ratio.

Further adjuvant studies were carried out with QA-7, QA-17, and QA-18 at different protein/adjuvant ratios. In general, a good adjuvant effect was observed for QA-7, QA-17, and QA-18 when used at protein/adjuvant ratios (protein weight/carbohydrate weight) of approximately 3:1 to 9:1 (FIG. 12). QA-21 (tested in this study only at protein/carbohydrate weight of 6:1) also showed an adjuvant effect. However, it should be noted that the proper adjuvant to protein ratio for optimum immune response is a function of both the particular saponin adjuvant and the particular antigen used. Adjuvant association with antigen plays an important role in the mechanism of action of the saponin adjuvant effect. In the case of saponin binding to protein, hydrophobic interactions are the predominant factor. Hence, differences in hydrophobicity of the HPLC-purified adjuvants will affect the binding constant to hydrophobic proteins. In addition, the number of hydrophobic binding sites on the protein will also affect the ability to associate with saponin adjuvants. Hence, it is necessary to determine the optimum adjuvant dose for each individual adjuvant and antigen. Such optimization is within the skill of the art.

HPLC-purified adjuvants were also compared with Freund's complete adjuvant and were found to result in a similar level of immune response (FIG. 12, panel b).

EXAMPLE 10

Preparation of FELV Recombinant gp70R-delta

Inclusion Body Preparation

Recombinant *E. coli* clone R16-38 was grown in LB medium supplemented with 1% glucose and 0.1% casamino acids at 32° C. to an optical density (560 nm) of 0.4-0.6. The culture was then shifted to 42° C. and incubated for an additional 2 hours. At the end of this time the cells were collected by centrifugation at 4,000 g for 30 minutes, washed with 50 Tris HCl, pH 7.5, and finally resuspended in 200 ml 50 Tris HCl to which is added 1 ml 0.1M phenylmethylsulfonylfluoride in isopropanol (final concentration 0.5 and 0.4 ml of 5 mg/ml aprotinin (final concentration = 10.0 ug/ml). The cells were lysed by enzymatic digestion with lysozyme (final concentration = 0.5 mg/ml) in the presence of 0.2% Triton X-100. After stirring for 30 minutes, 2 ml $MgCl_2$ (0.5M), 5 ml DNaseI (1 mg/ml) and 1 ml 0.1M phenylmethylsulfonylfluoride were added. After stirring for 30 additional minutes, 40 ml EDTA (0.25M, pH 7.5) and 4 ml Triton X-100 (10% w/v) were added. The preparation was centrifuged at 10,000 × g for 30 minutes at 4° C., and the pellet was resuspended in 50 ml 50 Tris HCl, pH 7.5. The pellet was homogenized at low speed for 15 seconds. Lysozyme was added to a concentration of 0.5 mg/ml and 0.6 ml of 10% Triton X-100 were added. After stirring for 15 minutes, 10 ml of $MgCl_2$ (0.5M) and 1 ml DNase I (1 mg/ml) were added and stirring was continued for an additional 15 minutes. After adjusting the volume to 300 ml with 50 Tris, pH 9.0, 40 ml of 10%

Triton X-100 and 51.2 ml of EDTA (0.25M, pH 7.5) were added and the final volume adjusted to 400 ml with 50 Tris, pH 9.0. After stirring for 30 minutes, the suspension was centrifuged at 10,000×g for 30 minutes at 4° C., and the pellet was resuspended in 400 ml 50 Tris HCl, pH 7.5, containing 4M urea, 50 EDTA, and 1% Triton X-100. After stirring for 15 minutes, the suspension was centrifuged at 10,000×g for 30 minutes at 4° C., and the pellet was resuspended in 400 ml 50 Tris HCl, pH 7.5, containing 1.0M NaCl. After stirring for 15 minutes, the suspension was centrifuged at 10,000×g for 30 minutes at 4° C., and the pellet was resuspended in 400 ml 50 Tris HCl, pH 7.5, containing 6M urea, and 5 EDTA. After stirring for 15 minutes, the suspension was centrifuged at 10,000×g for 30 minutes at 4° C. At this point the pellet of inclusion bodies was either frozen for future use or solubilized in 50 Tris HCl, pH 9.5, containing 6M guanidine HCl, 50 EDTA, and 0.5% beta-mercaptoethanol. The gp70R-delta polypeptide was then purified by either of the methods of Example 11, below.

EXAMPLE 11

Purification of FeLV Recombinant gp70R-delta

Procedure I

The solubilized protein of Example 8 was dialyzed against 6M urea, 50 Tris-Cl, pH 8.0, 5 EDTA, and 1 dithiothreitol (DTT). Approximately 120 mg of the protein was applied to a CM-TSK column (EM Science, 1.5 cm ID×4 cm) equilibrated with the same buffer. The protein was eluted with a linear gradient of NaCl (0–1.0M in 150 ml) in the same buffer. The fractions were collected and analyzed by electrophoresis on 10% SDS-polyacrylamide gels. Coomassie-staining was used to identify the gp70R-delta protein. Fractions 25–31, eluting at approximately 0.1M NaCl, were pooled and used for immunization.

Procedure II

In order to decrease the hydrophobicity of gp70R-delta, the sulfhydryl groups were alkylated with iodoacetamide and the lysine residues were N-acylated with citraconic anhydride. The protein prepared as in Example 8 was solubilized in 6M guanidine-HCl in 50 mM borate, pH 9.0, 0.5% beta-mercaptoethanol (v/v). Iodoacetamide is added at a molar ratio of 1:1 (iodoacetamide:total sulfhydryl groups). The alkylation was carried out in the dark for 1 hour at room temperature. The alkylation of all sulfhydryl groups (in the protein and beta-mercaptoethanol) was monitored with DTNB (Ellman's reagent) to ensure complete alkylation. The protein concentration was adjusted to 2 mg/ml.

The protein was citraconylated in the dark by the addition of citraconic anhydride (0.0022 ml per mg protein; approximately 50 molar excess over free lysines). The preparation was dialyzed several times in the dark against 50 mM borate, pH 9.0. The completion of the acylation of the protein lysine groups was determined by reaction with trinitrobenzene sulfonic acid (TNBS) which measures residual free lysine groups. TNBS (200 µl of 10 mM) was added to 200 µg alkylated, citraconylated, dialyzed gp70R-delta in 1 ml 50 mM sodium borate, pH 9.0. The mixture was incubated for 2 hours in the dark at 40° C., the reaction quenched with 0.5 ml of 1N HCl and 0.5 ml 1% SDS, and the absorbance was read at 340 nm. The concentration of TNP-lysine was determined using a molar extinction coefficient of 10,400.

The purification of the alkylated, citraconylated gp70R-delta was performed at pH 9.0 to prevent deblocking of lysine groups. Urea at a final concentration of 4M was added to the modified protein. The protein was concentrated to 3 mg/ml by ultrafiltration and applied to a Sepharose 6B-Cl column (1.5×86 cm). The gp70R-delta protein was eluted at a flow rate of 6.6 ml/hr with 4M urea, 50 mM sodium borate, pH 9.0. Fractions (5.3 ml/fraction) were collected and the gp70R-delta was determined by protein assay and SDS-polyacrylamide electrophoresis to be in fractions 13–15.

The citraconylation of gp70R-delta was reversed by dialyzing 5 ml of alkylated, citraconylated gp70R-delta (1.0 mg/ml) against 6M urea in 50 mM sodium citrate, pH 5.5 for 48 hours at room temperature. The gp70R-delta was dialyzed against 6 M urea in 100 mM sodium bicarbonate, pH 8.0 and the protein concentration adjusted to 0.8 mg/ml prior to absorption to aluminum hydroxide.

Procedure III

A modification of the above purification of alkylated, citraconylated gp70R-delta was developed. Briefly, alkylated, citraconylated gp70R-delta is modified and dialyzed against 50 mM sodium borate, pH 9.0 as described above. Urea was added to a final concentration of 8.0M. The protein was concentrated by ultrafiltration with a PM-30 membrane to yield 2.5 mg protein/ml. The protein solution was applied to a Sephacryl S-400 column (1.5×90 cm) in a 50 mM sodium borate buffer, pH 9.0 containing 8M urea and eluted with the same buffer. Fractions (2.9 ml/fraction) were collected and fractions 34–37 containing gp70R delta were pooled. Twenty-one mg of the protein from these fractions were diluted to a final concentration of 4M urea with 50 mM sodium borate, pH 9.0 and applied to a DEAE-TSK column (1.5×11 cm). The protein was eluted with a linear gradient of NaCl (0–0.5M) in 50 mM sodium borate, pH 9.0 containing 4M urea. Three ml fractions were collected. Fractions 89–95 containing gp70R-delta were pooled and 15 mg of gp70R-delta was recovered.

EXAMPLE 12

Immunization with Aluminum Hydroxide-Absorbed gp70R-delta

Aluminum hydroxide which has been found to have an adjuvant effect for many proteins and is coolly used in vaccines was used as a carrier for gp70R-delta. gp70Rdelta prepared by procedure I of Example 11 above absorbs tightly to 10% aluminum hydroxide in the presence of 50 mM Tris-Cl, pH 8.0 containing 6M urea. Approximately 3 µg gp70R-delta were absorbed per 100 µg aluminum hydroxide. The gp70R-delta absorbed to the aluminum hydroxide was washed with phosphate buffered saline (PBS), resuspended in PBS and used for immunization of animals.

CD-1 mice (8–10 weeks old) were immunized intradermally with gp70R-delta absorbed to $Al(OH)_3$ in a total volume of 200 µl PBS in the presence and absence of HPLC-purified saponins QA-17 or QA-18 or a mixture of QA-17 and QA-18. Twenty to twenty-five µg of gp70R-delta were injected per dose. HPLC-purified saponins QA-17 or QA-18 or a mixture of QA-17 and QA-18 were used at a dry weight dose of 10 µg. Two mice were injected for each formulation. Mice were given a booster injection of gp70R-delta/aluminum

hydroxide six weeks after the initial injection. Mouse sera was analyzed for reactivity to FEA, a FeLV subgroup A, at 2, 4, and 8 weeks post-immunization by an ELISA immunoassay. Four weeks following immunization, an anti-FeLV response elicited by the recombinant gp70-delta was observed. HPLC-purified saponin adjuvants QA-17 and QA-18 boost this response. The response was two orders of magnitude greater at four weeks post-immunization in the presence of QA-17 compared to immunization in the absence of saponin adjuvant. The results of this experiment are shown in FIG. 13.

Anti-FEA antibody was assayed by an ELISA assay. FEA virus (10 μg/ml in PBS) was absorbed to Immulon II plates overnight at 4° C. (100 μl/well). The plates were washed with PBS and nonspecific antibody binding was blocked by incubation for 1 hour with 10% normal goat serum in PBS (100 μl/well) at room temperature. Plates were then washed with 0.05% Tween-20 in distilled water. Sera was diluted in 10% normal goat serum in PBS and incubated for 1 hour at room temperature on the plate at serum dilutions of 10, 10^2, 10^3, and 10^4 (100 μl/well). After washing the plates with 0.05% Tween-20 in distilled water, they were incubated for 30 minutes at room temperature with 100 μl/well of peroxidase-conjugated goat anti-mouse IgG (Boehringer-Mannheim) diluted 1/5000 in PBS. After washing the plates with 0.05% Tween-20 in distilled water, the amount of IgG-binding was determined by peroxidase reaction with 3,3',5,5'-tetramethylbenzidine from the absorbance at 450 nm determined on a Dynatech microliter plate reader.

EXAMPLE 13

Immunization with Aluminum Hydroxide-Absorbed Alkylated gp70R-delta

CD-1 mice (8–10 weeks old) were immunized intradermally with 15 μg/dose of alkylated gp70R-delta purified by procedure II of Example 11 (absorbed to aluminum hydroxide as described in Example 12) in 200 μl PBS. HPLC-purified adjuvants AQ-7, AQ-17, AQ-18 and mixtures of the three adjuvants were used at a dry weight dose of 10 μg. Three mice were injected for each formulation. Mouse sera was analyzed by ELISA at 2 and 4 weeks postimmunization for reactivity to FEA as described in Example 10. As with immunization with unmodified gp70R-delta shown in Example 10, immunization with alkylated gp70R-delta elicits an anti-FeLV viral response by four weeks post-immunization. HPLC-purified adjuvants QA-7, QA-17, QA-18 all increase the immune response as compared to immunization in the absence of the saponin adjuvants. QA-17 and mixtures of QA-17 and QA-18 induced the highest response, inducing endpoint titers almost two orders of magnitude greater than immunization in the absence of saponin adjuvants. The results of these experiments are summarized on FIG. 14.

EXAMPLE 14

Toxicity of QA-7, QA-17, QA-18, QA-19, QA-21, "Quil-A"

With crude Quillaja saponins, a major symptom of toxicity in mice appears as necrosis of the liver. Purified saponins were injected into mice to determine effects on the liver. Mice were injected intradermally with 150 μg each QA-7, QA-17, QA-18, QA-21 and "Quil-A", the crude saponin extract used as the raw material for the purification of the other components. Animals injected with QA-7, QA-17, QA-18, and QA-21 appeared mildly ill initially but appeared to recover fully within a few hours after injection. "Quil-A" caused severe symptoms which continued for 48 hours. All mice were sacrificed at 48 hours for post-mortem examination of the liver. "Quil-A" caused severe damage of the liver with multifocal areas of acute necrosis evident. QA-7, QA-17, QA-18, and QA-21 did not seem to significantly affect the liver. QA-17 and QA-18 were also tested in kittens with subcutaneous injection of 100 μg each at 8 and 10 weeks, with no toxicity observed clinically or in the blood chemistry. In contrast, "Quil-A" induced a pyrogenic response which persisted for several hours in kittens. Hence, the purified saponins appear to be less toxic than "Quil-A" in both mice and kittens indicating that the purification process separates these saponins from one or more toxic components present in a crude Quillaja extract. One such toxic component has tentatively been identified as QA-19; dosages of 50 μg or greater were lethal in mice within a few days of injection. Further purification of QA-19 indicated that it could be separated into two peaks, QA-19a and QA-19b. QA-19a was lethal in mice at doses of 100 μg or greater whereas QA-19b was apparently nonlethal up to dose of 150 μg; hence, a synergistic effect to produce increased toxicity in the mixture of QA-19a and QA-19b cannot be ruled out. Preliminary screening of other minor peaks isolated from "Quil-A" indicates that other fractions may also be toxic. Hence, the purification protocols allow the separation of adjuvant-active saponins from similar but distinct compounds which are more toxic or which cochromatograph with toxic contaminants.

EXAMPLE 15

QA-18H and QA-21H, prepared as described in Example 7, were tested for adjuvant effect with BSA in direct comparison with the unhydrolyzed original products QA-18 and QA-21 prepared as described in Examples 3 and 4. QA-18 and QA-21 increase the humoral immune response to BSA in mice by at least an order of magnitude by two weeks post-immunization. However, the hydrolysis products QA-18H and QA-21H at the same weight dosage do not increase the response significantly (FIG. 15). Hence, optimal adjuvant effect is observed with the intact saponins; the essential structure required for adjuvant activity is lost or altered when QA-18 and QA-21 are hydrolyzed to QA-18H and QA-21H, respectively.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth below.

What is new and intended to be covered by Letters Patent of the United States is:

1. Substantially pure saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by a single predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, when analyzed on reverse phase-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID × 25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/mixture, and wherein said saponin has immune adjuvant activity and is less toxic when used as an adjuvant than said *Quillaja saponaria* extract.

2. Substantially pure QA-7 saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by one predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, and having a retention time of approximately 9-10 minutes when analyzed on reverse phase HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/minute.

3. The substantially pure QA-7 saponin of claim 2, wherein said saponin has immune adjuvant activity, and wherein said saponin is characterized by a carbohydrate content of about 35% per dry weight as assayed by anthrone, has a UV adsorption maxima of 205-210 nm, has a micellar concentration of 0.06% (w/v) in water and 0.07% in phosphate buffered saline, and causes no detectable hemolysis of sheep red blood cells at concentrations of 200 μg/ml.

4. The substantially pure QA-7 saponin of claim 3, wherein said carbohydrate content has a composition comprising the monosuccharides: terminal rhamnose, terminal xylose, terminal glucose, terminal galactose, 3-xylose, 3,4-rhamnose, 2,3-fucose, 2,3-glucuronic acid and apiose.

5. Substantially pure QA-21 saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by one predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, and having a retention time of approximately 51minutes when analyzed on reverse phase-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/minute.

6. The substantially pure QA-21 saponin of claim 5, wherein said saponin has immune adjuvant activity, and wherein said saponin is characterized by a carbohydrate content of about 22% per dry weight as assayed by anthrone, has a UV absorption maxima of 205-210 nm, has a micellar concentration of about 0.03% (w/v) in water and 0.02% (w/v) in phosphate buffered saline, and causes hemolysis of sheep red blood cells at concentrations of 25 μg/ml or greater.

7. The substantially pure QA-21 saponin of claim 6, wherein said carbohydrate content has a composition comprising the monosaccharides: terminal rhamnose, terminal arabinose, terminal apiose, terminal xylose, 4-rhamnose, terminal glucose, terminal galactose, 2-fucose, 3-xylose, 3,4-rhamnose and 2,3-glucuronic acid.

8. A substantially pure QA-17 saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by one predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, and having a retention time of approximately 35 minutes on reverse phase-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mN acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/minute.

9. The substantially QA-17 saponin of claim 8, wherein said saponin has immune adjuvant activity, and wherein said saponin is characterized by a carbohydrate content of about 29% per dry weight as assayed by anthrone, has a UV absorption maxima of 205-210 nm, has a micellar concentration of about 0.06% (w/v) in water and 0.03% (w/v) in phosphate-bufferred saline, and causes hemolysis of sheep red blood cells at concentrations of 25 μg/ml.

10. The substantially pure QA-17 saponin of claim 9, wherein said carbohydrate content has a composition comprising the monosaccharides: terminal rhamnose, terminal xylose, 2-fucose, 3-xylose, 3,4-rhamnose, 2,3-glucuronic acid, terminal glucose, 2-arabinose, terminal galactose and apiose.

11. A substantially pure QA-18 saponin purified from a crude *Quillaja saponaria* extract wherein said pure saponin is characterized by one predominant peak which comprises 90% or more of the total area of all peaks of a chromatogram, excluding the solvent peak, and having a retention time of approximately 38 minutes on reverse phase-HPLC on a Vydac C_4 column having 5 μm particle size, 330 Å pore, 4.6 mm ID×25 cm L in a solvent of 40 mM acetic acid in methanol/water (58/42; v/v) at a flow rate of 1 ml/minute.

12. The substantially QA-18 saponin of claim 11, wherein said saponin has immune adjuvant activity, and wherein said saponin is characterized by a carbohydrate content of about 25-26% per dry weight as assayed by anthrone, has a UV absorption maxima of 205-210 nm, has a micellar concentration of 0.04% (w/v) in water and 0.02% (w/v) in phosphate-buffered saline, and causes hemolysis of sheep red blood cells at concentrations of 25 μg/ml.

13. The substantially pure QA-18 saponin of claim 12, wherein said carbohydrate content has a composition comprising the monosaccharides: terminal rhamnose, terminal arabinose, terminal apiose, terminal xylose, terminal glucose, terminal galatose, 2-fucose, 3-xylose, 3,4-rhamnose and 2,3-glucuronic acid.

14. A method of enhancing an immune response to an antigen in an individual comprising administration of an amount of the substantially pure saponin adjuvants from any of claims 1-7 and 8-13 to said individual in an amount sufficient to enhance the immune response of said individual to said antigen.

15. A pharmaceutical composition useful for inducing the production of antibodies to an antigen in an individual comprising an immunogenically effective amount of an antigen and at least one substantially pure saponin as in any one of claims 1-7 and 8-13, wherein said substantially pure saponin is present in an amount sufficient to enhance the immune response of said individual to said antigen.

16. The pharmaceutical composition of claim 15, wherein said individual is a mammal.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,057,540

DATED : October 15, 1991

INVENTOR(S) : Charlotte A. Kensil, et al

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 10, line 32, "40 nM" should read "40 mM".

Column 11, Table 2, last line, under the heading "HPLC Fraction" there should be a dash " - " to denote that no fraction was included as adjuvant with the antigen.

Column 12, line 1, "QA-17" should read "QA-7".

Column 20, line 49, "coolly" should read "commonly".

Column 21, line 32, "microliter" should read as "microtiter".

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,057,540

DATED : October 15, 1991

INVENTOR(S) : Charlotte A. Kensil, et al

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 21, lines 41 and 42, "AQ-7, AQ-17, AQ-18" should read "QA-7, QA-17, and QA-18".

Column 23, line 22, "monosuccharides" should read "monosaccharides".

Column 24, line 1, "mN" should read "mM".

Signed and Sealed this

First Day of June, 1993

Attest:

Michael K. Kirk

MICHAEL K. KIRK

Attesting Officer

Acting Commissioner of Patents and Trademarks

EXHIBIT 8

0022-1767/91/1477-2136$02.00/0
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Print

EVALUATION OF MONOPHOSPHORYL LIPID A (MPL) AS AN ADJUVA

Enhancement of the Serum Antibody Response in Mice to Polysaccharide-Pr Conjugates by Concurrent Injection with MPL

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Concurrent injection of monophosphoryl lipid A (MPL) in saline or as an oil-in-water emulsion enhanced both the primary and secondary serum antibody responses to the capsular polysaccharide (CP) components of seven conjugates: the enhanced responses were Ag-specific. In contrast, MPL did not enhance the serum antibody response to five of the six unconjugated CP. MPL and trehalose dimycolate injected concurrently with the unconjugated Vi CP of *Salmonella typhi* (Vi) enhanced the serum antibody response to that Ag. MPL further enhanced the Vi antibody levels when injected with conjugates of this CP. The serum antibody responses to *Pseudomonas aeruginosa* exotoxin A, used as the carrier p2rotein for the *Staphylococcus aureus* types 5 and 8 conjugates, were also enhanced by MPL. MPL in oil-in-water emulsion was generally more effective than when administered in saline.

Immunogenelty is increased and T cell-dependent properties are conferred upon saccharides covalently bound to proteins to form conjugates (1). Further enhancement of antibody responses by adjuvants to medically important PS[2], such as the CP of Hib, could increase the percent of infants responding with protective levels of antibody to the first injection of conjugate and could reduce the dosage and the number of injections required to induce protective immunity to meningitis and other systemic infections caused by Hib (2–8). Adjuvants could also further enhance antibody responses induced by conjugates in patients with decreased immune responsiveness, such as occurs in sickle cell anemia, chronic renal hemodialysis, or with less-than-optimal nutrition (1, 9).

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] Abbreviations used in this paper: PS, polysaccharide; CP, capsular polysaccharide; MPL, monophosphoryl lipid A; Pn, pneumococcus; TDM, trehalose dimycolate; o/w, oil in water emulsion; Vi, Vi capsular polysaccharide; TT, tetanus toxoid; DT, diphtheria toxoid; ETA, *Pseudomonas aeruginosa* exotoxin A; Hib, *Haemophilus influenzae* type b; Pn3, pneumococcal type 3; GM, geometric mean.

Adsorption onto aluminium salts enhances a longs the antibody response to bacterial and v teins. Although this method enhanced the serun tibody responses of Hib-TT in infant rhesus mon adsorption onto aluminium salts did not serv adjuvant for Hib alone or an Hib-TT conjugate in old children (6).

MPL was shown to enhance the antibody resp Pn3 CP in adult and young mice (10–15). The mec was proposed to be inactivation of Ag-specific sup T cells by MPL (10, 11, 13). MPL in saline exer adjuvant action only when given after administr the Pn3 (11). Both the i.p. route and the inabilit MPL to augment the antibody response when a tered concurrently with the Pn3 limit the usefu this regime for humans.

In this study, we show that PS antibody resp 4- to 6-wk-old mice are enhanced by concurrent istration of MPL with these conjugates. Addition (ene to form an o/w emulsion with MPL and the co was generally more effective than MPL in sa adjuvant effect was also shown when TDM wa porated in an o/w emulsion.

MATERIALS AND METHODS

Antigens (Table I)

The preparation and immunologic properties of the CP and conjugates have been described (1, 3, 17–20). The dos conjugate was based by the amount of CP. Unless specif wise, each injection of Ag contained 2.5 μg of CP alon conjugate. The PS/protein ratios for the conjugates were Hib-TT (3), 0.5 for Pn6B-TT (9), 1.1 for Pn12F-DT, 0.66 : 1.28 for Vi-CTB, 1.37 for S. aureus type 5-ETA and 1.1 f ylococcus aureus type 8-ETA.

Adjuvants

The adjuvants were prohduced by RIBI ImmunoChem Inc, Hamilton, MT. MPL heptoseless mutant of *Salmonel sota* (R595) (21–23). TDM was prepared from cell walls of *terium phlei* by a modified method (24). This material h tectable low polarity lipids, mycolic acids, or trehalose d lates.

Vaccine Formulation

The CP or the conjugates were formulated with adjuva of two ways.

o/w. MPL and TDM were solubilized in chloroform/meth at 10 mg/ml. Aliquots were transferred to a 10 ml Potter

TABLE I

CP and CP protein conjugates[a]

Immunogen	Reference
Hib CP	(25)
Hib-TT	(3)
Pn6B, Pasteur, Mericux, France	(9, 27)
Pn6B-TT	(3, 9)
Pn12F, Lederle Laboratories, NY	(27)
Pn12F-DT	(17)
VI, Pasteur Mericux, Paris, France	(1)
VI-cholera toxin-(VI-CT)	(19)
VI-B subunit cholera toxin (VI-CTB)	(20)
S. aureus type 5 and type 8 CP	(1, 18)
S. aureus types 5 and 8-*P. aeruginosa* ETA	(18)

[a] The polysaccharides and proteins are covalently bound by multipoint attachment using either adipic acid dihydrazide (3, 9) or *N*-succinimidyl 3-(2-pyridyldithio)propionate as the joining reagents (17–20). All the conjugates were excluded from the matrix by gel filtration through CL-4B Sepharose.

tissue grinding tube with a fitted Teflon pestle. The chloroform/methanol was evaporated by passing N_2 into the tube. An aliquot of squalene (Sigma Chemical Co., St. Louis, MO) passed through a sterile 0.22-nm filter, representing 2% of the final volume of vaccine, was added to the tube. The contents were ground with a motor-driven pestle until the MPL and/or TDM were solubilized in the oil (~3 min). The Ag solution was added to the tube and the mixing continued for 2 to 4 min. Tween 80 (Sigma) 0.2% in saline was added and this mixture was homogenized by up-and-down strokes of the pestle for 4 to 6 min. Thimerosal was added to a final concentration of 0.01% and the vaccine was decanted into vials and stored at 4°C. Before use, the vaccines were warmed to room temperature and vortexed. In certain experiments (see *Results*) o/w contained MPL only or both MPL and TDM. Control o/w contained only squalene and water.

Admixture with MPL in saline. The freeze-dried triethyleneamine (Sigma) salt of MPL was solubilized at 1.0 mg/ml in 0.25% triethyleneamine by heating at 50°C for 15 min followed by sonication (Branson 1200, Branson Ultrasonics Corp., Danbury, CT) for 15 min. The resulting solution was slightly opalescent. Aliquots of this solution were then admixed with the Ag in a 5-ml vial and the formulation brought to a final volume with saline, 0.01% thimerosal, and stored at 4°C.

The dose of Ag was varied in some experiments (see *Results*) but the dose of adjuvant was held constant in all experiments: 50 μg of MPL in an o/w or in saline or 50 μg each of MPL and TDM in o/w.

Immunization of Mice

Groups of 20 ICR (Harlan Sprague Dawley) female mice, 4 to 6 wk of age, were injected s.c. with 0.1 ml of vaccine. After 14 days, 10 mice from each group were exsanguinated. The remaining mice in each group were injected again with the same dose on day 14 and exsanguinated 7 days later. The sera were stored at −70°C. The sera from each group, as well as from controls (injected with saline) were coded and sent to the NIH for antibody assay.

Serologic

CP antibodies were measured by RIA for Hib (25), Pn6B, Pn12F, and the VI (19, 21). ELISA was used to measure antibodies to the *S. aureus* type 5 and type 8 CP and to the ETA of *P. aeruginosa* (18).

Statistical

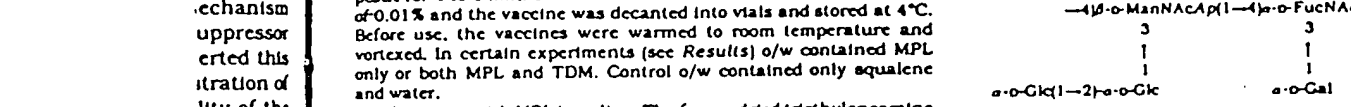
The antibody responses of postimmunization sera were expressed as GM levels. Comparison between groups were calculated using the unpaired *t*-test. All computations were performed using the Statistical Analysis System.

RESULTS

Hib-TT. The Hib CP is a linear homopolymer with the following repeat unit: →3)β-D-Rib*f*(1→1)ribitol-5-PO_4-. Injection of Hib CP alone, with MPL, or with MPL and TDM did not elicit antibodies in the mice (data not shown). Consistent with our published results (2, 3 TT elicited serum Hib CP antibodies in mice. Addi MPL + TDM, as an o/w, enhanced the serum H antibody responses after the first injection (Tal Although GM Hib antibody levels in the adjuvant g are higher than in the Hib-TT alone group, these ences were NS after the second injection.

The effect of these adjuvants was studied with v doses of Hib-TT (Table III). In our previous experir one-tenth of a human dose was injected into m evaluate the immunogenicity of Hib-TT and other gates designed for clinical use (1). Although not a statistically significant, there was a direct relati tween the dose of Hib-TT and the GM after each inje Addition of the adjuvants to the 0.10 and 0.5 μg dc Hib-TT enhanced the Hib antibody response aft second injection. The GM Hib antibody levels in adjuvant groups were higher than those elicited Hib-TT alone.

Pn6B-TT and Pn12F-DT (Table IV). Both Pn6 Pn12F are components of the 23 valent pneumo vaccine (27). Pn6B is a linear copolymer whose tet charide repeat unit is composed of: →)α-D-Gal*p*(1→ Glc*p*(1→3)α-L-Rhap(1→4)-D-ribitol-5-PO_4-. Pn12F linear branched chain copolymer whose repeat he charide unit is:



Pn6B and Pn12F CP are structurally and antige unrelated. Pn6B alone did not elicit a rise in homo antibodies neither with MPL in saline or in o/w. elicited a slight rise in homologous antibodies afte injection (NS). Neither the CP alone or with MPL a heterologous serum antibody response.

Conjugates of these two CP elicited statistically icant antibody rises with a booster after the second

TABLE II

Concurrent injection of Hib-TT conjugate with 50 μg of MPL/T an o/w

Immunogen	Geometric Mean μg Ab/ml (25–75th Centiles)	
	Post 1st injection	Post 2nd injec
Hib-TT	0.33[a] (0.12–0.58)	1.46 (0.16–1
Hib-TT + MPL + TDM	1.35[a] (0.55–3.14)	6.75 (0.91–:
MPL + TDM	0.04 (0.03–0.04)	0.16 (0.03–(
Saline	0.04 (0.03–0.04)	0.04 (0.03–(

[a] *b* vs *a*: $p = 0.04$.

TABLE III

Effect of MPL + TDM injected as an o/w concurrently with v doses of Hib-TT

μg Hib-TT injected	Geometric Mean μg Ab/ml (Nonresponders[a])			
	Hib-TT alone		Hib-TT + MPL + TI	
	Post 1st injection	Post 2nd injection	Post 1st injection	Post injec
0.10	0.19 (5)	0.23[a] (4)	0.48 (2)	2.03
0.50	0.16 (4)	0.50[a] (3)	0.79 (1)	4.86
2.50	0.33 (2)	1.64 (0)	1.43 (0)	4.48
12.5	0.71 (0)	1.23 (0)	2.03 (0)	3.11
Saline	<0.06	<0.06		

[a] Nonresponders are those with <0.15 μg antibodies/ml in the 10 mice (25).

c vs *b*: $p = 0.009$, *e* vs *d*: $p = 0.002$.

TABLE IV

Serum antibodies elicited in mice by s.c. injection of pneumococcal types 6B or 12F CP alone or as conjugates with MPL in saline or in o/w

Vaccine Formulation	GM Titer ng Ab/ml (no. > 300 ng Antibodies/Total)			
	Pneumococcus type 6B		Pneumococcus type 12F	
	1st injection	2nd injection	1st injection	2nd injection
Pn6B-TT	28.1 (1/10)	336.0[e] (4/10)	0.2 (0/10)	0.6 (0/10)
Pn6B-TT + MPL	56.5[a] (2/10)	813.0[g] (9/10)	5.9 (0/10)	6.3 (0/10)
Pn6B-TT + MPL, o/w	331.0[b] (8/10)	2704.0[f] (10/10)	7.4 (1/10)	3.6 (0/10)
Pn6B	17.3 (2/10)	0.0 (0/10)	2.9 (0/10)	0.0 (0/10)
Pn6B + MPL	23.6 (0/10)	3.0 (0/10)	3.4 (0/10)	18.8 (0/10)
Pn6B + MPL, o/w	5.1 (0/10)	13.2 (0/10)	11.8 (0/10)	11.4 (0/10)
Pn12F-DT	5.6 (0/10)	27.3 (0/10)	24.3[j] (1/10)	343.8[k] (9/10)
Pn12F-DT + MPL	13.3 (0/10)	36.2 (0/10)	153.6[p] (1/10)	401.7[l] (7/10)
Pn12F-DT + MPL, o/w	10.3 (0/10)	48.5 (0/10)	218.8[n] (4/10)	603.0[i] (8/10)
Pn12F	9.5 (1/10)	6.2 (0/10)	4.4 (0/10)	23.5 (0/10)
Pn12F + MPL	5.7 (0/10)	24.5 (0/10)	52.0 (0/10)	84.8 (1/10)
Pn12F + MPL, o/w	1.6 (0/10)	9.5 (0/10)	17.8 (1/10)	25.2 (1/10)
Saline	4.4 (0/10)	0.7 (0/10)	14.9 (0/10)	1.1 (0/10)
Saline	11.5 (0/10)	5.4 (0/10)	81.4 (1/10)	11.3 (0/10)

b vs *a*; $p < 0.05$, *e* vs *d*, *g* vs *f*: $p = 0.05$, *h* vs *f*: $p = 0.02$, *e* vs *c*: $p = 0.0007$, *g* vs *h*; NS, *i* vs *h*, *g*, *f*: $p = 0.01$.

tion ($p = 0.001$) (3, 17). The addition of MPL enhanced the antibody responses to both CP ($p = 0.05$): MPL as an o/w elicited higher antibody levels than MPL in saline ($p = 0.05$). Neither the conjugates alone nor with the adjuvants elicited a rise in heterologous antibodies.

Vi conjugates (Table V). The Vi, a linear homopolymer of (1→4) αGalNAc*A*p variably *O*-acetylated at C3, elicits serum antibodies and protects mice against mucin-enhanced lethal infection with *Salmonella typhi*: reinjection does not elicit a booster response (28). Addition of o/w to the Vi had no effect on antibody formation elicited by the Vi. Addition of MPL alone or with TDM in o/w enhanced the Vi antibody response after both primary and secondary immunizations ($p < 0.05$).

Neither the Vi-CTB nor the Vi-CT elicited enhanced Vi antibody responses after the first injection. Both conjugates elicited a booster response after the second injection: Vi-CT was more immunogenic than the Vi-CTB ($p < 0.01$). Addition of the o/w alone enhanced slightly (NS) the response of the Vi-CTB but not the Vi-CT. Addition of MPL or MPL plus TDM to the o/w enhanced the serum Vi antibody rises elicited by both conjugates after both injections with the enhancement of Vi-CT > Vi-CTB ($p < 0.001$). The increment of vaccine-induced Vi antibodies elicited by the adjuvants in o/w over the Vi conjugate alone was the highest in our study.

TABLE V

Serum Vi antibodies elicited by Vi, Vi conjugates alone or with MPL, alone or with MPL and TDM in an o/w

Vaccine	ng Vi Ab/ml GM (Range)	
	Post 1st injection	Post 2nd injection
Vi	4.41[a] (2.38–7.38)	7.09[i] (1.62–73.2)
Vi + o/w	4.10[a] (2.61–7.29)	2.99[d] (1.61–6.77)
Vi + MPL + o/w	11.5[e] (2.80–42.0)	14.8[e] (6.10–30.6)
Vi + MPL + TDM + o/w	15.8[b] (0.95–229)	21.6[f] (12.2–41.4)
Vi-CT	2.52[a] (1.20–3.92)	31.8[i] (16.5–56.0)
Vi-CT + o/w	4.18[a] (2.42–6.44)	61.2[j] (30.8–131)
Vi-CT + MPL + o/w	8.30[b] (3.14–31.8)	206[l] (103–724)
Vi-CT + MPL + TDM + o/w	12.4[b] (6.14–46.8)	526[l] (33.9–2230)
Vi-CTB	2.20[a] (1.04–9.22)	9.75[c] (0.90–89.4)
Vi-CTB + o/w	2.69[a] (0.99–10.3)	22.2[c] (0.90–89.4)
Vi-CTB + MPL + o/w	23.2[j] (6.65–143)	30.9[c] (9.78–115)
Vi-CTB + MPL + TDM + o/w	48.3[j] (34.6–199)	106[p] (7.05–299)
Saline	0.07 (0.03–0.68)	0.07 (0.03–0.16)

b vs *a*, *h* vs *g*, *o* vs *m*: $p < 0.05$, *f* vs *c*: $p = 0.01$, *e*, *f* vs *d*, *l* vs *k*, *p* vs *n*, *p* vs *o*: $p < 0.01$, *i* vs *j*: $p = 0.0001$.

Vi conjugates alone was the highest in our study.

S. aureus types 5 and 8—P. aeruginosa ETA conjugates. S. aureus type 5 and type 8 CP are linear copolymers with a trisaccharide repeat unit containing an *O*-acetylated ManNAcA (1, 18):

Type 5: →4)β-D-ManNAcp*A*p(1→3)α-L-FucNAcp(1→3)β-D-FucNAcp(1→
3
|
OAc

Type 8: →3)β-D-ManNAcA*p*(1→3)α-L-FucNAcp(1→3)α-D-FucNAcp(1→
4
|
OAc

Neither the type 5 nor the type 8 CP alone or with MPL or with MPL + o/w elicited serum antibodies (Table VI). Both type 5 and type 8 CP conjugates of ETA elicited a rise in serum antibodies after the first injection and a booster response. Addition of MPL to the conjugates enhanced the CP antibody response ($p = 0.001$ for type 5-ETA and $p = 0.0001$ for type 8-ETA). Addition of o/w to the type 5-ETA conjugate further enhanced the primary and secondary antibody responses to the CP ($p = 0.01$). This further enhancement, however was not observed with the *S. aureus* type 8-ETA MPL + o/w.

Addition of MPL to the conjugates enhanced the level of serum ETA antibodies. The ETA antibody levels elicited by the conjugates with MPL in o/w were not different from those elicited by the conjugates with MPL in saline.

DISCUSSION

Different proteins and synthetic schemes were used to prepare the seven conjugates. The addition, the immunogenicity of the CP differs: for example, Pn6B is a comparatively poor immunogen for mice and man, whereas the Vi elicits protective levels of antibodies in both species (1, 19, 28). Inasmuch as the adjuvants in this study enhanced Pn6 CP antibody production, it is probable that MPL o/w may serve as an adjuvant for

TABLE VI

Serum antibodies elicited in mice by two injections of S. aureus CPs alone or conjugated to ETA as conjugates with MPL or with MPL in

Vaccine Formulation	Type 5		ETA		Vaccine Formulation	Type 8		ETA	
	1st injection	2nd injection	1st injection	2nd injection		1st injection	2nd injection	1st injection	2r injec
Type 5-ETA	2[a]	17[b]	<1[f]	5[k]	Type 8-ETA	10[l]	149[q]	2[r]	12
Type 5-ETA + MPL	14[c]	59[d]	4[g]	163[t]	Type 8-ETA + MPL	42[m]	750[s]	7[t]	40
Type 5-ETA + MPL, o/w	5[e]	134[e]	<1[f]	39[u]	Type 8-ETA + MPL, o/w	34[n]	555[u]	5[v]	16
Type 5	2	3	ND	<1	Type 8	1	2	ND	N
Type 5 + MPL	2	3	ND	<1	Type 8 + MPL	2	2	ND	N
Type 5 + MPL, o/w	1	3	ND	<1	Type 8 + MPL, o/w	2	ND	ND	N
Saline	1	2	ND	ND					

b, e vs a: p = 0.0001. d vs b, c: p = 0.03. k, h vs f: p = 0.0001. t vs g: p = 0.0005. k, t vs h: p < 0.001. l vs k: p = 0.03. o vs l, P vs m, q v
0.0001. P, q vs o: p < 0.01. u, t vs r: p = 0.0001. t vs s: p = 0.03.

other PS-protein conjugates.

The adjuvant effect was less pronounced for Hib-TT than for the other conjugates. Although not always statistically significant, higher antibody responses were observed in all the groups receiving these adjuvants compared to the Hib-TT alone.

We have used bacterial toxoids as carriers for our conjugates (1). Inasmuch as these proteins may elicit protective antibodies, it is necessary to assay the immune response to these Ag to ascertain that the adjuvants did not exert a deleterious effect on their immunogenicity. In this study, only antibodies to the *P. aeruginosa* ETA were evaluated. MPL in saline or as an o/w enhanced the antibody response to ETA compared to the conjugates alone. Whereas the MPL o/w was a more effective adjuvant for the *S. aureus* CP antibodies, the ETA antibodies were higher in the MPL in saline group. In deciding which formulation is more immunogenic, the effect on the carrier protein will have to be evaluated.

We have not studied whether administration of MPL 2 days later exerts an adjuvant effect on PS antibody synthesis induced by these conjugates. It is unlikely that any regimen for adjuvants, aside from their simultaneous administration with the vaccine, would be suitable for immunization of humans, especially infants. In these experiments, the MPL o/w or saline was admixed with the conjugate and required some mechanical agitation before injection. This procedure is suitable for immunization programs. We have also studied the adjuvant effects of MPL in o/w on *Shigella dysenteriae* type 1-TT, planned for clinical evaluation.[3] The MPL + o/w enhanced serum antibody levels in mice to the O-specific polysaccharide of this conjugate after primary and secondary immunizations.

MPL in liposomes or combined with mycobacterial cell wall skeleton in o/w emulsions have been evaluated in adult volunteers both as adjuvants alone for treatment of malignancies or combined with peptide-protein conjugates as vaccines for malaria (23, 29, 30). No serious side effects were encountered in these studies. Young, outbred mice have served as a reliable model to compare the immunogenicity of conjugates to the CP alone (1–3, 9, 17, 31). Our data, therefore, support clinical evaluation of MPL on the immunogenicity of these conjugates in the population at risk.

[3] Chu, C. Y., S. C. Szu, D. C. Watson, J. B. Robbins, and R. Schneerson. *Manuscript in preparation.*

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EXHIBIT 9

with a twofold increase in serum creatinine, followed by death from pulmonary oedema. These pathophysiological events are characteristic of lethal, hypodynamic sepsis[12,13].

Passive immunization of baboons with anti-cachectin antibody infused 1 h before bacterial challenge conferred beneficial cardiovascular effects (Fig. 1) but not complete protection against critical organ injury (Table 1). In contrast to the controls, blood pressure did not fall acutely after bacteraemia, but was maintained by a compensatory increase in heart rate (up to 175 beats per min) and cardiac output. Although acute cardiovascular collapse and shock were not observed, serious renal injury did occur, as evidenced by twofold increases of serum creatinine at 8 h and the development of anuria. Each of the baboons immunized 1 h before bacterial challenge developed fatal pulmonary oedema.

Should uniform distribution and tissue penetration of the antibody not have occurred within 1 h, we investigated protection using earlier passive immunization with anti-cachectin $F(ab')_2$. When antibody was administered 2 h before *E. coli*, normal blood pressure was maintained during bacteraemia, significant tachycardia occurred only transiently, and shock was prevented. Neither did vital organ dysfunction occur or renal failure develop, and there was no evidence of pulmonary oedema. Animals recovered from anaesthesia, were active and healthy, and resumed eating within 24 h. No evidence of persisting sepsis or tissue injury was observed by physical examination or routine complete blood count until the time that animals were killed for necropsy. Thus, early passive immunization with monoclonal anti-cachectin antibodies protected against the effects of bacterial sepsis.

These results with anti-cachectin antibodies were not due to increased bacterial clearance in the immunized animals, or to bacteriocidal properties of the antibody solution: the viability of circulating bacteria 2 h after *E. coli* infusion was similar in the control ($3.1 \pm 0.5 \times 10^4$ CFU per ml) and immunized baboons ($3.9 \pm 0.8 \times 10^4$ CFU per ml), and decreased comparably in both groups within 4 h ($1.3 \pm 0.6 \times 10^3$ CFU per ml). During this period, the inhibition of cachectin by $F(ab')_2$ resulted in improved cardiac output, suggesting that cachectin is necessary to provoke septic shock. The administration of $F(ab')_2$ alone without bacterial challenge did not significantly change blood pressure, cardiac output or hormone counter-regulatory release over the 10-h monitoring period. Recovery from anaesthesia was uneventful, normal activity and food intake was resumed overnight and post-mortem examination was normal. The improved cardiovascular function in the antibody-treated bacteraemic animals cannot be attributed to differences in hydration status, because similar amounts of fluid were administered to all animals in accordance with a predetermined protocol (data not shown).

During sepsis, the systemic release of catabolic stress hormones in part mediates the maintenance of cardiovascular tone and mobilization of host energy stores[15]. We observed persistent increases in circulating adrenaline, noradrenaline, and glucagon in all animals succumbing to bacterial challenge (Table 2), but early pre-treatment with antibodies blunted the magnitude of later (8 h) counter-regulatory hormone responses in all survivors. We have previously shown that the infusion of recombinant cachectin produces hypotension and diminished cardiac output despite endogenous catecholamine production (which would normally increase cardiac output)[10]. As antibody administration is associated with improved cardiac output, the present data also suggest that sepsis-associated myocardial depression is due in part to cachectin.

It appears then that the normal injurious host responses elicited by overwhelming bacteraemia can be prevented if the effects of cachectin are blocked. The death of the infected host could result from an overexpression of cachectin during a normally beneficial immune response, somewhat analogous to anaphylactic shock, during which protective immune responses are capable of inducing shock and death. It is probable that the provocative toxicity of cachectin arises in part from a direct effect on normal tissues, and in part from the release of other humoral factors. Complement activation, release of the interleukins, interferons and platelet-activating factor, and the induction of other cytokines would be expected to amplify and broaden the range of host responses[16-19].

Experimental models of endotoxaemia and sepsis perhaps differ from the indolent and progressive syndrome of multiple system organ failure in human patients, but cachectin has been implicated in human infections[20,21]. Levels of cachectin are frequently elevated during acute meningococcal sepsis, when those patients with the highest cachectin levels die[22]. The prophylactic use of anti-cachectin antibodies in patients at high risk for overwhelming infection could protect against septic shock. Further experiments are needed to determine whether the administration of anti-cachectin antibodies during persisting infection or indolent sepsis will be beneficial.

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γ Interferon, CD8⁺ T cells and antibodies required for immunity to malaria sporozoites

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This study was designed to test the hypothesis that T-cell effector mechanisms are required for protective immunity to malaria sporozoites. Administration of neutralizing monoclonal antibodies against γ interferon (γIFN) to immune hosts, reversed sterile immunity to sporozoite challenge, by allowing the growth of

exoerythrocytic forms (EEF) and thus the development of parasitaemia. Immune animals also developed infections when depleted *in vivo* of their suppressor/cytotoxic T cells expressing the CD8 antigen ($CD8^+$) but not when depleted of helper T cells expressing CD4 antigen ($CD4^+$), before sporozoite challenge. Passive transfer of immune immunoglobin alone, or adoptive transfer of immune T cells alone, conferred partial protection to naive recipients. Transfer of both immune components resulted in significantly greater protection. This transferred immunity was reversed by the *in vivo* neutralization of γIFN. Thus, sterile immunity to sporozoite challenge requires the neutralization of sporozoites by antibodies and the inhibition of EEF development by γIFN with the participation of $CD8^+$ cells.

Vaccination of hosts with attenuated malaria sporozoites confers protective immunity to viable sporozoite challenge[1]. This immunity is mediated in part by antibodies to the circumsporozoite (CS) protein[2-4]. As immunity can be induced in B-cell depleted mice[5], however, T cells may also contribute to protection. It is difficult, however, to envisage cellular mechanisms which could neutralize a large sporozoite inoculum in the short time before hepatocyte invasion.

Recent studies show that a novel T-cell mediated immune mechanism may act not against sporozoites, but against the developing liver-stage parasites: recombinant γIFN at very low concentrations inhibits the development of EEF within hepatocytes, *in vivo*[6,7] and *in vitro*[8]. In this study, we tested the hypothesis that γIFN, released from sensitized T cells during sporozoite challenge, inhibits the development of EEF, and is required for sterile immunity. We also investigated whether helper or cytotoxic T cells are required for immunity to sporozoite challenge.

Immunized rats were challenged with *Plasmodium berghei* sporozoites. Following challenge, the animals received either monoclonal antibody (mAb) DB-1, which neutralizes rat and mouse γIFN[9], or an irrelevant control mAb. After 44 h the level of EEF development was determined by DNA hybridization[10]. As shown in Fig. 1, naive animals receiving DB-1 or the control mAb showed similar high levels of EEF growth. As expected, immunized animals receiving the irrelevant mAb showed no detectable levels of EEF DNA. Immunized animals receiving mAb DB-1, however, showed substantial levels of EEF development, that is, 43.5% of control levels with inocula of 2.5×10^4 or 5×10^4 sporozoites. A similar proportion of EEF was rescued from destruction by endogenous γIFN activity in immune hosts when DB-1 was administered 2.5 h after challenge. As sporozoites can invade hepatocytes within 2 min of inoculation[11], and are cleared from the circulation within 30 min[12], the targets of endogenous γIFN are indeed the developing intrahepatocytic EEF.

We extended our experiments to determine whether *in vivo* neutralization of γIFN, or *in vivo* depletion of immune T cells, would reverse sterile immunity and allow the development of blood-stage parasites. Mice that had been immunized according to established protocols remained protected when challenged with 5×10^3 *P. berghei* sporozoites, followed by 1 mg of irrelevant control mAb. Blood-stage infection, however, developed in immunized animals that received 1 mg of mAb DB-1 either immediately or 2.5 h after challenge (Table 1, Expt 1). In another experiment, mice which had been hyperimmunized with repeated injections of large numbers of X-irradiated sporozoites resisted challenge doses of 2×10^4–1.5×10^5 sporozoites. In parallel experimental groups receiving DB-1 after challenge with 1.5×10^5, 10^5 or 5×10^4 sporozoites, most animals developed blood-stage parasitaemia. The anti-sporozoite-antibody titres in the hyperimmunized groups were high (24,576) which may account for their resistance to the lower challenge dose of 2×10^4 sporozoites (Table 1, Expt 2).

γIFN can be produced by helper and suppressor/cytotoxic T cells. To determine if either T-cell subset is involved in mediating protection, groups of immunized mice received MAbs with lytic activity for $CD4^+$ or $CD8^+$ T cells[13]. As shown in Table 2, the elimination of $CD8^+$ T cells in fully immune animals rendered them susceptible to challenge with 5×10^3 sporozoites. In contrast, destruction of $CD4^+$ T cells did not reverse host immunity.

Table 1 Effect of γIFN neutralization on immunity to sporozoite challenge

Immune status	Challenge dose	No. infected/total		Day of patency (range)		Reciprocal IFA titre
		DB-1	Control mAb	DB-1	Control mAb	
Expt 1						
Naive	5,000*	5/5	5/5	4.0 (4.0)	4.0 (4.0)	32
Immune	5,000*	5/5	0/5	5.8 (4–7)	—	6,144
Immune	5,000†	4/5	0/5	6.0 (5–7)	—	6,144
Expt 2						
Naive	20,000	5/5	5/5	4.0 (4.0)	4.0 (4.0)	32
Hyperimmune	20,000	0/5	0/5	—	—	24,576
Hyperimmune	50,000	2/5	0/5	6.5 (6–7)	—	24,576
Hyperimmune	100,000	4/5	0/5	6.75 (6–7)	—	24,576
Hyperimmune	150,000	4/5	0/5	5.5 (3–6)	—	24,576

Neutralization of endogenous γIFN reverses immunity to sporozoite challenge. Antibody titres of pooled sera determined by IFA using glutaraldehyde-fixed sporozoites. Mice were challenged with variable doses of sporozoites. Test groups received either control mAb or mAb DB-1. The appearance of parasitaemia (patency) was determined by daily examination of thin blood films. Experiment 1; test groups immunized with 6×10^4 sporozoites followed by two boosts of 10^4 sporozoites at two-week intervals; * received mAbs immediately after challenge; † received mAbs 2½ h after challenge. Experiment 2; test groups hyperimmunized with 10^5 sporozoites followed by four boosts of 10^5, 5×10^4, 5×10^4 and 5×10^4 sporozoites at three-week intervals. Naive controls and test groups received mAbs immediately after challenge. Comparison of control and treatment groups in both experiments showed a significant level of heterogeneity ($P < 0.01$), by Kruskal-Wallis test. The level of significance was adjusted using the Bon-Ferroni transformation.

Passive and adoptive transfer experiments were undertaken to clarify the relative contributions of humoral and cellular mechanisms to protective immunity. Naive mice received purified IgG or splenic T lymphocytes from immune or naive donors, or a combination of immune IgG and T cells. In contrast to previous studies[14,15], the mice did not receive booster immunizations with irradiated sporozoites after transfer of spleen cells. As shown in Table 3, control mice which received either nonimmune IgG or naive T lymphocytes soon developed parasitaemia. Those receiving immune IgG alone, or immune T lymphocytes alone, were partially protected against infection, that is, the prepatent period was significantly prolonged, indicating a substantial reduction in the infectivity of sporozoites or in the development of EEF. Mice receiving both immune IgG and immune T lymphocytes showed the greatest degree of protection (60%). This transferred immunity was reversed by administration of mAb DB-1 to recipients ($P < 0.001$). Clearly, challenge with a small number of sporozoites (10^3), similar to

Table 2 Effect of $CD8^+$ depletion on immunity to sporozoite challenge

Immune status	No. infected/total	Mean day of patency (range)
Immunized, untreated	0/10	—
Immunized, $CD4^+$ depleted	0/5	—
Immunized, γIFN depleted	4/5	5.25 (5–6)
Immunized, $CD8^+$ depleted	5/5	4.4 (4–5)
Naive	5/5	4.8 (4–5)

Depletion of $CD8^+$, but not $CD4^+$, T cells reverses immunity to sporozoite challenge. Immune animals were inoculated intravenously with mAbs with lytic activity against $CD8^+$ or $CD4^+$ T cells, or DB-1, and were challenged with 5×10^3 *P. berghei* sporozoites. The degree of T-cell subset depletion *in vivo* was determined by surface immunofluorescence assay on splenic lymphocytes from parallel T-cell depleted and control animals (92% and 94% depletion for $CD8^+$ and $CD4^+$ subsets, respectively).

Table 3 Passive and adoptive transfer of immunity to sporozoites and EEF

Treatment	No. infected/total	Mean of patency (range)	Reciprocal IFA titre
Expt 1			
Non-immune T cells	5/5	4.2 (4–5)	16
Immune T cells	5/5	5.8 (5–6)	32
Immune T cells +mAb DB-1	5/5	4.2 (4–5)	32
Expt 2			
Nonimmune IgG	4/5	4.0 (4)	64
Non-immune T cells	5/5	4.2 (4–5)	32
Immune IgG	4/5	5.5 (5–6)	2,048
Immune T cells	4/5	5.75 (4–7)	128
Immune Ig +immune T cells	2/5	7.5 (7–8)	3,072
Immune Ig + immune T cells +mAb DB-1	5/5	5.0 (5)	3,072

Donor A/J mice were immunized with X-irradiated sporozoites. Affinity-purified serum IgG and splenic T lymphocytes (2×10^7) purified on Lymphocyte M-separation medium (Accurate), followed by panning on affinity-purified goat anti-mouse IgG-coated plates, were inoculated intravenously into naive recipient A/J mice 36 h before challenge with 10^3 (Expt 1) or 3.5×10^3 (Expt 2) viable sporozoites. Some groups received mAb DB-1, or control mAb, immediately following challenge. Patency and antibody titres were determined as described (Table 1). Comparison of control and treatment groups was by Kruskal–Wallis test ($P < 0.01$). $P < 0.001$ for comparison of the differences in patency between recipients of both immune antibodies and immune T cells, and recipients of immune antibodies, immune T cells and mAb DB-1, by Mann–Whitney (Wilcoxon) test.

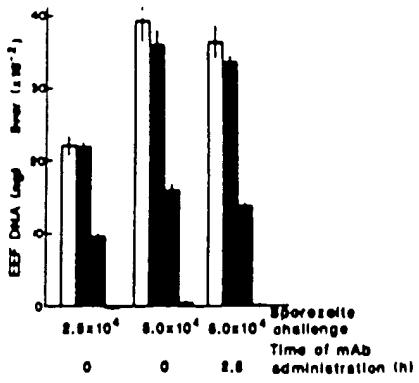

Fig. 1 Endogenous γIFN inhibits development of EEF in immune hosts. Rats immunized with four doses of 10^4 X-irradiated sporozoites of *P. berghei*, at biweekly intervals, were challenged after three weeks with 2.5×10^4 or 5×10^4 sporozoites. Immediately or 2½ h later, animals received an intravenous inoculation of 1 mg of mAb DB-1, or a control mAb. EEF-DNA levels were determined after 44 h, as described[10]. Data are mean values from five animals ± s.d. □, Naive plus DB-1; ▨, naive plus control mAb; ▩, immunized plus DB-1; ■, immunized plus control mAb.

that inoculated by the bite of infected mosquitoes, is sufficient to induce a γIFN response (Table 3, expt 1).

$CD8^+$ T cells may exert their effect in two ways. They may respond to the presentation of parasite antigens in the context of Class I major histocompatibility complex (MHC) glycoproteins with the production of γIFN which then acts in a hormonal fashion against EEF. Binding of the lymphokine to surface receptors on infected hepatocytes destroys EEF *in vitro* without the participation of immune effector cells[8]. In support of this interpretation, the protection afforded by adoptive transfer of splenic T cells was reversed by mAb DB-1 (Table 3). In addition $CD8^+$ T cells may be directly cytotoxic for infected hepatocytes which present processed antigens of sporozoite or EEF origin.

Although some sporozoites can evade anti-sporozoite antibodies and become established within the host liver (Fig. 1), γIFN alone may not be sufficient to provide sterile immunity, as very high levels are required to eradicate EEF[6–8]. Thus, whereas each component may be insufficient to mediate complete protection, they can act additively (Table 3). In another study, rodents were immunized with a synthetic peptide corresponding to the repeat domain of the CS protein of *P. berghei*. High levels of antibodies to sporozoites were induced without detectable peptide-specific cellular responses. Extensive protection was observed upon challenge with a small number of sporozoites[16]. Thus the relative contribution of humoral and cellular mechanisms to sporozoite/EEF immunity depends upon the interaction of at least three variables, namely the titre of neutralizing antibodies, the degree and type of cellular response to parasite antigen and the challenge dose of viable sporozoites itself, and will therefore vary with choice of antigen and method of vaccination.

As *in vivo* destruction of $CD4^+$ T cells did not affect host immunity, and $CD4^+$-intact, $CD8^+$-deleted animals were susceptible to challenge, the MHC Class II restricted T-helper epitopes required for antibody production to the CS protein[17–20] are not sufficient to prime T cells for γIFN mediated protection. $CD8^+$ cells recognize MHC Class I restricted epitopes, which may be structurally distinct. Our results suggest that effective human malaria sporozoite vaccines will require the incorporation of synthetic peptide or recombinant protein analogues of parasite B, T-helper, and T-cytocytoxic epitopes, capable of inducing high titres of neutralizing antibodies to the CS protein, and of sensitizing T-cell subsets that respond with the production of γIFN to parasite antigens presented during natural sporozoite challenge.

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EXHIBIT 10

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Immunology

CD8+ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites

(*Plasmodium yoelii*/CD4+ T-helper cells)

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ABSTRACT In recent malaria sporozoite vaccine trials in humans and mice, antibodies to the sporozoite coat protein have given only modest protection against sporozoite challenge. In contrast, irradiated sporozoites can protect mice against massive sporozoite infections. Evidence suggests that immunity in these mice is mediated by T cells. To identify the mechanism of immunity, we used monoclonal antibodies specific for either the CD4 or CD8 molecule to selectively deplete sporozoite-immunized mice of T-cell subsets. Though *in vivo* depletion of CD4+ T cells did not reduce immunity, depletion of CD8+ T cells abolished protection. Monoclonal antibody treatment did not affect anti-sporozoite antibody levels. Our data indicate that cytotoxic T cells are critical for immunity to large numbers of sporozoites and suggest that vaccine development should be reoriented toward stimulating cellular as well as humoral immunity.

In the life cycle of the malaria parasite, sporozoites pass from the mosquito through the blood of the host and invade liver cells where they develop into hepatic-stage parasites. Later, mature parasites are released from the liver to invade erythrocytes. When sporozoites are experimentally irradiated, they still invade liver cells but they are unable to mature to the stage that infects erythrocytes (1). Immunization with irradiated sporozoites can protect mice against infection with several thousand sporozoites (2). Humans can also be successfully immunized with irradiated sporozoites (3, 4), but use of attenuated parasites as a vaccine is impractical. A current sporozoite vaccine strategy is to induce antibodies against the central repetitive sequence of the circumsporozoite (CS) protein, which covers the sporozoite surface (5–7). Several recent trials of such vaccines in humans (8, 9) and mice (10, 11) have had limited success. This suggests that humoral immunity may be less important than previously thought and that cellular immunity may be critical for a highly effective vaccine (10, 12, 13). Indeed, μ-suppressed mice, which lack B cells and circulating immunoglobulins, can be immunized with sporozoites (14), demonstrating that T cells are sufficient for sporozoite immunity.

In this paper we dissect the T-cell response to malaria sporozoites by depleting immune mice of T cells carrying either the CD4 or CD8 surface molecule. CD4 and CD8, previously referred to in the mouse as L3T4 and Lyt2, respectively, are molecules that function together with the T-cell receptor complex (15, 16). Outside of the thymus, most T cells carry only one of these molecules that restrict T-cell activation. T cells carrying the CD8 molecule can only be activated by antigens presented along with class I major histocompatibility complex (MHC) molecules, whereas T cells bearing the CD4 molecule are activated by antigens along with class II MHC molecules (17). In animals, CD4-bearing T cells function mainly as T-helper cells, whereas CD8-bearing T cells are mainly cytotoxic and suppressor cells (18). The *in vivo* injection of monoclonal antibodies to CD8 or CD4 results in the almost complete depletion of T cells with these markers from the spleen and lymph nodes (19). We have used these antibodies to eliminate T-cell subsets in mice immune to malaria sporozoites to determine which T cells are responsible for anti-sporozoite immunity.

MATERIALS AND METHODS

Mice. BALB/c and athymic mice were purchased from Charles River Breeding Laboratories.

Sporozoites. *Plasmodium yoelii* (17X NL) sporozoites in *Anopheles stephensi* mosquitoes were harvested 14–16 days after an infectious blood meal. Harvesting was by a modification of the method of Ozaki *et al.* (20). Thirty to 60 mosquitoes were anesthetized with chloroform and placed on a glass slide in a drop of medium 199 with 3% mouse serum. The abdomen was held with fine forceps, and the thorax of each mosquito was cut just anterior to the wing. The collection of upper bodies without further preparation was then spun through glass wool according to the published method. After counting in a hemacytometer, sporozoites were diluted to final concentration in medium 199 with 3% mouse serum.

Antibodies. Monoclonal antibodies were produced in ascites fluid of athymic mice and of BALB/c mice treated with cortisone and irradiation (21). Anti-CD8 antibody came from the anti-Lyt2.2 hybridoma 19/178 (mouse IgG2a) (22). Anti-CD4 antibody came from the anti-L3T4 clone GK1.5 (rat IgG2b) (23). A control antibody came from the anti-*Plasmodium falciparum* gamete clone 1B3 (mouse IgG2a) (45). Control rat immunoglobulin was from normal rat serum purchased from Accurate Chemical and Scientific (Westbury, NY). All immunoglobulins were purified by 50% ammonium sulfate precipitation. The rat anti-CD4 antibody and rat serum immunoglobulin were further purified over an anion-exchange column. Final antibody concentrations were determined by optical density or by enzyme-linked immunosorbent assay (ELISA).

Immunization Protocol. Mice 4–26 weeks old were immunized with three or four doses of live sporozoites that had received irradiation (10,000 rads; 1 rad = 0.01 gray) from a ^{137}Cs source. The first immunization was of 75,000 sporozoites; subsequent immunizations were of 20,000 sporozoites given at 2- to 4-week intervals. All immunizations were administered into a tail vein. Two to 4 weeks after the final immunization, animals were challenged with 5000 in-

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Abbreviations: CS, circumsporozoite; IFA, indirect immunofluorescence antibody; SRBC, sheep erythrocyte(s); PFC, plaque-forming cell(s); MHC, major histocompatibility complex; FMF, flow microfluorometry; FITC, fluorescein isothiocyanate.

Table 1. Effect of anti-T-cell treatments on sporozoite immunity

Immune status	Treatment	Experiment 1* Infected/total	Experiment 1* Days to detectable parasitemia: Median	Experiment 1* Days to detectable parasitemia: Range	Experiment 2† Infected/total	Experiment 2† Days to detectable parasitemia: Median	Experiment 2† Days to detectable parasitemia: Range
Normal	None	10/10	4	4–5	5/5	5	5–7
Immune	None	0/4	Not detected		0/6	Not detected	
Immune	Anti-CD8 mouse IgG2a	5/5	6	5–6	4/4	5	5–6
Immune	Control mouse IgG2a	ND			0/5	Not detected	
Immune	Anti-CD4 rat IgG2b	ND			0/5	Not detected	
Immune	Control rat serum IgG	ND			0/5	Not detected	

ND, not done.
*Mice received three doses of irradiated sporozoites; blood smears were taken beginning on the fourth day after challenge.
†Mice received four doses of irradiated sporozoites; blood smears were taken beginning on the third day after challenge.

fectious sporozoites administered through a tail vein and followed with blood smears for 14 days. This is a large dose, as the injection of 20 *P. yoelii* sporozoites causes infection half of the time in normal mice. Seventy-two of 74 animals were not infected and were considered immune. Immunity was stage specific, as sporozoite-immunized mice were susceptible to infection by transfusion of *P. yoelii*-infected blood. These immune animals were then used within 2–4 weeks in the studies described below.

Lymphocyte-Depletion Protocol. For $CD8^+$ T-cell depletion, immune mice were injected intraperitoneally (i.p.) with 1 mg of anti-CD8 antibody for 2 successive days and then challenged with 5000 infectious sporozoites by way of the tail vein 2 days later. As a control group, another set of immune mice received 1 mg of mouse IgG2a on the same schedule. For $CD4^+$ cell depletion, immune mice received 0.5 mg of anti-CD4 antibody i.p. for 8 days and were challenged with 5000 sporozoites 2 days later. These mice received additional injections of 0.5 mg of anti-CD4 every 3 days. A control group of immune mice received 0.5 mg of rat immunoglobulin on the same schedule.

Flow Microfluorometry (FMF). The following reagents were used: fluorescein isothiocyanate-conjugated (FITC) anti-Thy1.2 (24) (Dupont/New England Nuclear); goat anti-mouse IgG2a FITC (Southern Biotechnology, Birmingham, AL); mouse-adsorbed goat anti-rat immunoglobulin FITC (Kierkegaard and Perry, Gaithersburg, MD); biotinylated anti-CD8 (clone 53-6.5) (24) (Becton Dickinson); Texas red-avidin and biotinylated anti-CD4 (clone H129.19) (25), gifts of B. J. Fowlkes. A FACS-440 flow cytometer (Becton Dickinson) was used for reading one- and two-color-stained samples. Spleen cells from antibody-treated mice were examined by FMF to quantitate T-cell depletion. Animals were sacrificed following treatment either (*i*) at the time of challenge, (*ii*) when parasites appeared in the peripheral blood, or (*iii*) 11 days after challenge if animals were protected. Single-color FMF was performed by using anti-CD8 (clone 19/178) followed by goat anti-mouse IgG2a FITC or anti-CD4 (clone GK1.5) followed by goat anti-rat immunoglobulin FITC. Two-color FMF was performed by using anti-Thy1.2 FITC (green channel) and either biotinylated anti-CD8 or biotinylated anti-CD4 with Texas red-avidin (red channel).

Plaque-Forming Cell (PFC) Assay. Mice were immunized i.p. with 0.2 ml of a 10% suspension of sheep erythrocytes (SRBC). They were killed 4 days later, and PFC assays were performed according to standard methods (26).

Serology. All animals were bled for serum after monoclonal antibody treatment on the day before sporozoite challenge. Sera were titered by ELISA (11) against a synthetic 24-amino acid peptide, $(Gln\text{-}Gly\text{-}Pro\text{-}Gly\text{-}Ala\text{-}Pro)_4$, corresponding to four copies of the *P. yoelii* CS protein 6-amino acid repeat. Pooled sera were also assayed by indirect immunofluorescent antibody assay (IFA) against air-dried *P. yoelii* sporozoites (27).

Parasitemia. Thin blood films were taken daily starting on the third or fourth day after sporozoite challenge. Blood films were Giemsa stained and 50 oil-immersion fields were scanned for parasites. Mice were considered to be protected if no parasites were detected by day 11 after challenge.

RESULTS

In two experiments we observed that *in vivo* depletion of CD8 T cells completely abolished sporozoite immunity (Table 1). In the first experiment anti-CD8 antibody-treated mice had a slightly longer time to detectable parasitemia than did control animals. In the second experiment no such delay was observed, with treated and control animals developing detectable parasitemia on day 5 (median). In contrast, all immune animals treated with anti-CD4 antibodies were still protected. Immune animals that received control antibodies were also protected from sporozoite challenge. Anti-sporozoite antibody titers by ELISA and IFA were similar in treated and control animals (Table 2).

FMF analysis was performed to quantitate T-cell depletion (Fig. 1). Animals were sacrificed and spleen cells were taken either at the time of challenge or following sporozoite challenge and assessment of immunity. The extent of immunodepletion was the same at both time points. An average of 94% of $CD8^+$ T cells was removed following anti-CD8 antibody treatment, and an average of 97% of $CD4^+$ T cells was removed following treatment with anti-CD4 antibody. Neither antibody depleted T cells of the other phenotype.

Because depletion of $CD4^+$ T cells had no effect on immunity, we wished to assess T-helper cell function in the depleted mice. Anti-CD4-treated mice, normal mice, and nude mice were given SRBC i.p., and 4 days later PFC responses were measured in spleens. As shown in Table 3,

Table 2. Effect of immunodepletion on anti-sporozoite antibodies

Immune status	Treatment	IFA	ELISA: Median	ELISA: Range
Normal	None	<1:10	<1:16	ND
Immune	None	1:560	1:256	1:64–1:1024
Immune	Anti-CD8 mouse IgG2a	1:560	1:512	1:32–1:1024
Immune	Control mouse IgG2a	1:560	1:256	1:128–1:1024
Immune	Anti-CD4 rat IgG2b	1:280	1:256	1:128–1:1024
Immune	Control rat serum Ig	1:560	1:512	1:256–1:1024

Serum was taken the day before sporozoite challenge from animals in experiment 2, Table 1. IFA was performed on pooled serum samples. ELISA was performed on each serum, and results are shown as the median and range for each group. ND, not done.

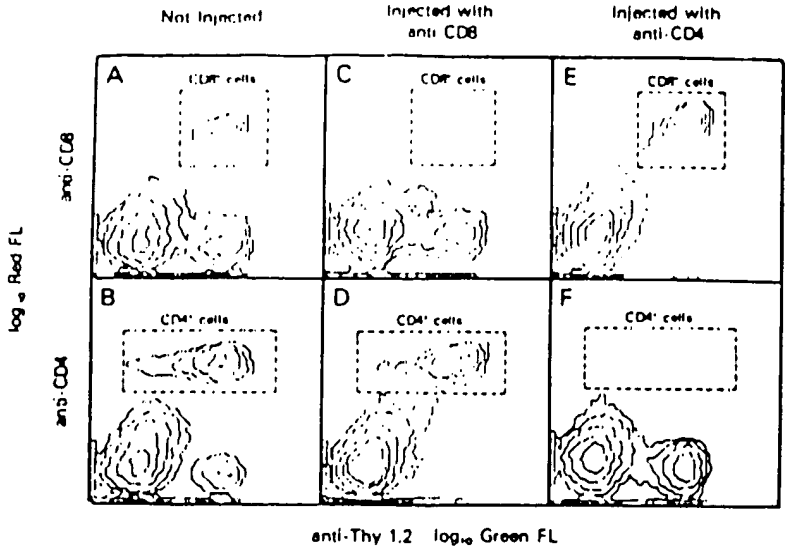

FIG. 1. Spleen cells from sporozoite-immunized mice (Table 1, experiment 2) analyzed by two-color FMF. (*A*) No anti-T-cell treatment, stained for CD8 and Thy1. $CD8^+$ cells are present. (*B*) Same sample as *A*, stained for CD4 and Thy1. $CD4^+$ cells are present. (*C*) Treated with anti-CD8 antibody, stained for CD8 and Thy1 on the day sporozoites produced a detectable parasitemia. $CD8^+$ cells are depleted. (*D*) Same sample as *C*, stained for CD4 and Thy1. $CD4^+$ cells are present. (*E*) Treated with anti-CD4 antibody, stained for CD8 and Thy1, 11 days after sporozoite inoculation. $CD8^+$ cells are present. (*F*) Same sample as *E*, stained for CD4 and Thy1. $CD4^+$ cells are depleted, but animals did not develop parasitemia. FL, fluorescence.

the PFC (IgM) responses of mice injected with anti-CD4 were below those of uninjected mice and were comparable to PFC responses of nude mice, confirming that T-helper cell function was absent.

DISCUSSION

Our data indicate that the $CD8^+$ subset of T cells is necessary for the protection of sporozoite-immunized mice against a challenge of 5000 infectious sporozoites. In contrast, another major subset, the $CD4^+$ T cells, is not required for a protective response in previously immune animals. These conclusions come from depleting live animals of T-cell subsets by using injections of monoclonal antibodies specific for known T-cell-surface molecules. $CD8^+$ T cells have primarily cytotoxic and suppressor cell functions, whereas $CD4^+$ T cells function primarily as T-helper cells (17, 18). Injection of mice with antibody to CD8 removes $CD8^+$ T cells (19) and has been shown to halt the rejection of tumors (28) and to interfere with the clearance of viral infections (29). Injection of antibody to CD4 clears $CD4^+$ T cells (19) and prevents delayed-type hypersensitivity responses (30) and antibody production to T-dependent antigens (31). As it is difficult to imagine how depleting a suppressor cell could reduce an immune response, we believe the critical $CD8^+$ T cells depleted in these experiments are cytotoxic T cells.

How could cytotoxic T cells kill malaria parasites in sporozoite-immunized mice? T cells recognize antigens only after they have been processed and presented on cell surfaces along with MHC molecules (32). Thus, T cells cannot act directly on the free-swimming sporozoite but respond to parasites after sporozoites enter host cells and processed parasite antigens are displayed on the cell membrane. $CD8^+$ T cells can only be activated by cells bearing class I MHC molecules, whereas $CD4^+$ T cells are restricted by class II MHC molecules (18). Since hepatocytes carry only class I MHC molecules (33) they can present parasite antigens only to $CD8^+$ T cells. $CD8^+$ T cells could kill infected hepatocytes by classic cell–cell cytotoxic mechanisms or by the local release of lymphokines. Interferon γ is known to kill hepatic-stage parasites (34–37) and is made by $CD8^+$ T cells (38). Although $CD4^+$ T cells can make interferon γ, they would not be triggered by the infected hepatocytes, which do not carry class II MHC molecules. This is consistent with our observation that anti-CD4 treatment does not alter the effector response of immune mice.

Table 1. Effect of anti-CD4 antibody treatment on the PFC (IgM) response to SRBC

Mice	Treatment	$CD4^+$ splenocytes, %	PFC Per 10^6 cells	PFC Per spleen
BALB/c	None	26.2	1364	261,141
BALB/c	Anti-CD4	0.4	15	2,025
Athymic	None	2.9	68	4,461

The results are the averages of two BALB/c mice without treatment, five BALB/c mice with anti-CD4 treatment, and two athymic nude mice. The percentage of splenocytes that was $CD4^+$ was calculated by single-color FMF.

If we wish to stimulate cellular responses with a synthetic anti-sporozoite vaccine, the first task is to identify the parasite antigens recognized by $CD8^+$ T cells. These antigens must have been presented to the host during immunization with irradiated sporozoites. However, they need not be from the CS or other sporozoite proteins and may be liver-stage antigens (1, 39). From indirect evidence, we speculate that the CS protein is indeed the target for the effector T cells. Any parasite antigen on the hepatocyte surface recognized by cytotoxic cells would be expected to come under selective pressure; parasites expressing mutations within these T epitopes would not be recognized and killed. A comparison of four sequences of the CS protein from different clones of *P. falciparum* (40) reveals that the polymorphic segments of the molecule correspond to the human immunodominant T-cell epitopes (44). This implies that these epitopes are important to parasite survival, and they may be the target antigens for cytotoxic T cells.

$CD8^+$ T cells are necessary for effector immunity in sporozoite-immunized animals, but alone they may not provide the optimal immune response. Ultimately, the best vaccine may induce humoral and T-cell immunity. Since cytotoxic T cells are generally not activated by exogenous protein antigens (32, 41, 42), other approaches such as recombinant viruses or liposomes (43) may need to be tried if synthetic vaccines are to mimic the cellular immunity stimulated by irradiated sporozoites.

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EXHIBIT 11

United States Patent [19]

Cantrell

[11] Patent Number: 4,877,611

[45] Date of Patent: Oct. 31, 1989

[54] VACCINE CONTAINING TUMOR ANTIGENS AND ADJUVANTS

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[21] Appl. No.: 102,909

[22] Filed: Sep. 30, 1987

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 852,120, Apr. 15, 1986, Pat. No. 4,806,352.

[51] Int. Cl.[4] A61K 39/39; A61K 39/04

[52] U.S. Cl. 424/88; 514/885; 514/937; 514/938; 514/934; 514/943

[58] Field of Search 424/88, 92; 514/885, 514/937, 938, 939, 943

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[57] ABSTRACT

Vaccines are provided which are composed of (a) non-toxic and highly effective adjuvants obtained from microbial sources, together with (b) tumor antigens. A wide variety of antigens can be employed in the vaccines and include, antigens obtained from tumors or cultures of tumor cells, such as ovarian cancers, melanomas, colorectal cancers, pancreatic cancers, renal cancers and the like. By adding tumor antigens to potent but non-toxic immunostimulants, a protective and lasting tumor immunity can be obtained.

22 Claims, No Drawings

VACCINE CONTAINING TUMOR ANTIGENS AND ADJUVANTS

This application is a continuation-in-part of U.S. application Ser. No. 852,120 now U.S. 4,806,352 filed Apr. 15, 1986 and which is incorporated hereby by reference.

FIELD OF THE INVENTION

This invention relates, in general, to tumor vaccines. In one aspect, this invention is directed to a vaccine composed of non-toxic and highly effective adjuvants from microbial sources and tumor associated antigens. In a further aspect, this invention is directed to a process for the preparation of tumor vaccines and to a method for their use in the treatment and prevention of tumors by enhancing the efficiency of immunogenic tumor antigens.

BACKGROUND OF THE INVENTION

Endotoxin has been recognized over the last ninety years as a potent immunoactivator. W. B. Coley reported in *Ann. Surg.* 14 199 (1891) the use of "Bacterial Toxins" in cancer treatment. It was also reported in *JAMA* 54 250 (1919) that the cure rate in inoperable patients utilizing such a mixed microbial vaccine was 4-7 percent. Endotoxin extracts were subsequently studied in several animal models. Gratia and Linz in *Comp. Rend. Soc. De Biol.* 108: 427 (1931) described hemorrhagic necrosis and concommitant tumor regression in a transplantable liposarcoma model in guinea pigs. Other rodent tumor models followed: sarcoma 180 in mice (Shwartzman, G. and Michailovsky, N., *Proc. Soc. Exp. Biol. Med.* 29: 737 (1932)), Ehrlich carcinomas in mice (Berendt, M. J. and North, R. J., Exp. Med., 151: 69 (1980)), and transplantable tumors in rats (Berendt, M. J. and North, R. J., private communication.).

Recent work on the observation of tumor necrosis in endotoxin-treated animals indicates that the endotoxic fraction itself may not be directly responsible for necrosis (Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Liore, N., and Williamson, B., *Proc. Nat'l Acad. Sci. USA* 72: 3666 (1975)). Instead, necrosis formation may be mediated by a factor termed tumor necrosis factor (TNF) which has been isolated from mice previously stimulated by macrophage activators such as BCG, *C. parvum* or zymosan. In addition, TNF has been shown to be cytotoxic against certain tumor lines in vitro, and antitumor activity has been ascribed to this substance in vivo.

Prior to the present invention and during a search for microbial components having antitumor activity, it was found that when certain preparations of endotoxin were combined with trehalose dimycolate (TDM) and oil droplets and injected into established malignant line-10 tumors in Strain 2 guinea pigs, a high rate of cures and systemic tumor immunity developed. This led to a reinvestigation of the value of endotoxin as an immunotherapeutic agent. The most powerful endotoxin adjuvants were phenol-water (PQ) or chloroform methanol (CM) extracts from Re (heptoseless) mutant, gram-negative bacteria. These extracts contained endotoxic lipopolysaccharides (LPS) which made up phenol-water extracts from wild-type bacteria. Both ReG1 and lipipolysaccharide when injected in combination with TDM and oil droplets caused a rapid developing Shwartzman-like necrotic reaction in the tumors. Following this reaction, the LPS combination led to only a partial regression of injected tumors, and their growth continued after about two weeks. In contrast, injection of the ReG1-TDM combination led to high rates of permanent regression and development of systemic immunity against a challenge with line-10 tumors. Tumor regression with ReG1+TDM or CWS+TDM and more advanced tumors could be treated with greater success.

In a paper by Dr. J. L. Cantrell et al appearing in *Cancer Research* 39, 1159-1167, April (1979) it was disclosed that a combination of chemotherapy and immunotherapy are highly effective in causing regression of an established tumor in mice, whereas either treatment alone was ineffective. In this study, the immunotherapy used involved injection of KCl - extracted tumor antigens in oil-in-water emulsions with or without trehalose dimycolate.

Also in U.S. Pat. Nos. 4,436,727 and 4,505,903 various combinations of refined detoxified endotoxin or purified pyridine soluble extracts of microorganisms with cell wall skeleton and/or trehalose dimycolate were disclosed as being useful in the treatment of cancerous tumors. However, prior to the present invention, immunotherapy was performed with biological response modifiers, as non-specific immunotherapy or with tumor antigens alone. However, non-specific immunotherapy had only a short effect on tumors and tumor antigens were low in immunogenicity. In addition, the adjuvants previously available for use in human vaccine had low activity, and hence the immunotherapy was not entirely satisfactory. Thus, while there is considerable prior art on adjuvants and on tumor antigens, there is no prior art on the use of non-toxic biological adjuvants to enhance protective immunity when used in combination with tumor antigens. Therefore, what was needed was a potent but non-toxic immunostimulant which could be utilized in conjunction with tumor associated antigens to provide a protective and lasting tumor immunity.

Accordingly, one or more of the following objects will be achieved by the practice of this invention. It is an object of this invention to provide a vaccine which is effective in thie treatment and prevention of tumors. Another object of this invention is to provide a vaccine comprised of non-toxic and highly effective adjuvants from microbial sources and tumor antigens. A further object of this invention is to provide a method for enhancing the antitumor activity of immunogenic tumor antigens. Another object of this invention is to provide a process for the preparation of tumor vaccines. A still further object is to provide a process for the preparation of tumor vaccines comprised of adjuvants and tumor antigens. Another object is to provide a process for the preparation of vaccines comprised of refined, detoxified endotoxins and tumor antigens. A further object is to provide a method for using the vaccines in the treatment and prevention of tumors. These and other objects will be readily apparent to those skilled in the art in light of the teachings contained herein.

SUMMARY OF THE INVENTION

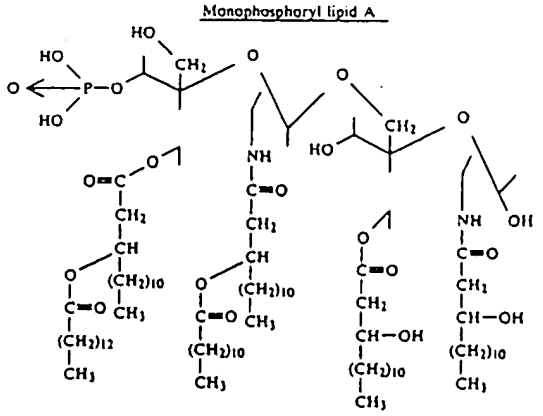
As hereinbefore indicated, the present invention is directed to vaccines comprised of non-toxic and highly active adjuvants obtained from microbial sources and tumor antigens. The invention also relates to processes for preparation of the vaccines and their use in the treatment and prevention of tumors.

The vaccines of the present invention are comprised of:

(a) at least one tumor associated antigen,

Monophosphoryl lipid A obtained from lipopolysaccharides of *Salmonella minnesota* R595 has been given as follows:

Monophosphoryl lipid A



(b) a refined detoxified endotoxin immunostimulant

(c) at least one additional immunostimulant selected from the group consisting of:

(1) microbacterial cell wall skeleton

(2) trehalose dimycolate, and

(3) a pyridine soluble extract of a microorganism, and

(d) a pharmaceutically acceptable carrier.

DETAILED DISCUSSION OF THE INVENTION

As indicated, the vaccines of the present invention are comprised of a tumor associated antigen hereinafter also referred to as (TAA), a refined detoxified endotoxin immunostimulant referred to as (MPL) and at least one other bacterial immunostimulant. The tumor associated antigens which are employed in the vaccines of the present invention can be whole cells, fraction of cells or extracts of tumor cells prepared by known techniques. In some instances it might be desirable to utilize two or more antigens in the same vaccine.

Illustrative tumor associated antigens include but are not limited to antigens obtained from warm-blooded animal tumors such as ocular carcinoma, sarcoid, ovarian cancer, mammary tumors, adenocarcinoma, pancreatic carcinoma, renal carcinoma, lung carcinoma and the like.

The refined detoxified endotoxin immunostimulant employed in the present invention is identified as monophosphoryl lipid A (MPL), and is prepared in the manner set forth in U.S. Pat. Nos. 4,436,727 and 4,436,728 which are incorporated herein by reference. Endotoxin extracts of the type used as the starting material to produce MPL may be obtained from any Enterobacteriaciae including parent organisms and mutants. The aforesaid patents describe the type of microorganisms that may be used to obtain the starting material and several methods for preparing the starting material. The detoxified endotoxin can also be prepared synthetically and by genetic engineering techniques. The preferred method to date of obtaining the endotoxic extract is that disclosed by Chen et al., J. infect. Dis. 128 543 (1973).

Monophosphoryl Lipid A (MPL), is a composition characterized as having no detectable 2-keto-3-deoxyoctonoate, between about 350 and 475 nmoles/mg of phosphorus and between about 1700 and 2000 nmoles/mg of fatty acids. The complete structure of a MPL is a significant improvement over endotoxic extracts obtained from Enterobacteriaciae because MPL is detoxified and therefore does not contain the highly toxic components which have rendered endotoxic extracts unsuitable for therapeutic use. (See *Peptides as Requirements for Immunotherapy of the Guinea-Pig Line-10 Tumor with Endotoxins;* Ribi, et al. Cancer Immunol. Immunother. Vo. 7, pp 43-58: 1979, incorporated herein by reference. The beneficial effects of MPL over other endotoxic extracts is described for example in U. S. Patents Nos. 4,436,727 and 4,436,728; and Ribi, E. Journal of Biological Response Modifiers. Vol 3, pp 1-9: 1984, incorporated herein by reference).

As indicated above, the endotoxin immunostimulant employed in the vaccines of the invention has been detoxified in accordance with the procedures set forth in U. S. Patents 4,436,727 and 4,436,728. By "detoxified" is meant that the toxicity (LD_{50} value) of the endotoxin, based on chick embryo lethality assay, is more than 20 micrograms when compared to the toxic endotoxin where the LD_{50} value is approximately 0.001 micrograms.

In addition to the monophosphoryl lipid A immunostimulant employed in the vaccine of the invention, at least one additional bacterial adjuvant is also present. As indicated above, such adjuvants include (a) mycobacterial cell wall skeleton, (b) trehalose dimycolate and (c) a pyridine soluble extract of a microorganism.

The first bacterial adjuvant which can be employed with the antigen and MPL is the cell wall skeleton which is essentially cell wall which has had much of the protein and lipids normally found in the cell wall removed. It is a polymeric mycolic acid arabinogalactan mucopeptide containing remnants of trehaiose mycolates ("P3") and undigested tuberculoproteins. Cell wall skeleton is obtained from any microorganism including, but not limited to, *M.smegmatis, M.phlei, Nocardia rubra, Nocardia asteroides, Corynebacterium diphtheria, Corynebacterium parvum, M.kansaii. M.tuberculosis* (Strain H 37 and RV and Ayoma B), and *M.bovis* Strain BCG. Additionally, cell wall skeleton may be obtained from such other organisms as *E.coli, B.abortus* and *Coxiella burnettii.*

Cell wall skeleton may be produced by first growing and harvesting bacteria such as *M.bovis* strain BCG (Bacillus Calmette-Guerin). The resulting whole cell residue is processed through a cell fractionator [Ribi Cell Fractionator (Sorvall. Model RF-1)] which disrupts the cells, separating the outer envelope or cell wall from the protoplasmic impurities. The resulting cell walls are then subjected to a series of solvent extractions and enzymatic treatments (e.g. trypsin and/or chymotrypsin) to give purified cell wall skeleton.

A second bacterial adjuvant which can be utilized in the vaccines of this invention are the trehalose dimycolates (TDM) which may be obtained from organisms such as, for example, *M.avium. M.phlei, M.tuberculosis* (strain H 37 RV and Ayoma B), *M.bovix* BCG, *M.smegmatis, M.kansaii, Nocardia rubra, M.bovinis* and *Corynebacterium diphtheriae.*

Bacteria such as *M.avium* are grown, harvested and then heat killed. The cell mass is then extracted with several solvents and then an active solvent soluble, fraction is extracted. This extract is further purified by a series of solvent extractions to provide crude TDM (see *Biologically Active Components from Mycobacterial Cell Walls.* 1. *Isolation and Composition of Cell Wall Skeleton and Component,* P. 3; Azuma et al., Journal of the National Cancer Institute, Volume 52, pgs. 95–101, 1974) incorporated herein by reference. As disclosed in Azuma et al., crude TDM may then be further purified by centrifugal microparticulate silica gel chromatography to give purified TDM. TDM may also be prepared as disclosed in U. S. Patent 4,505,900 which issued March 19, 1985.

The third bacterial adjuvant which can be included in the vaccines of the present invention is a pyridine-soluble extract of a microorganism containing between about 3 and 20% by weight of protein, between about 10 and 40% by weight of sugar and about 35 to 60% by weight of fatty acids. The extract preferably contains about 5% by weight of each protein, about 35% by weight of sugar and about 5% by weight of fatty acids.

The protein comprises amino acids and ammonia and the amino acids include, for example, the following:

Asparginine	0.273
Threonine	0.108
Serine	0.585
Muramic acid	0.219
Glutamic acid	0.267
Glycine	0.39
Alanine	0.173

The amounts expressed above are in terms of weight percent and the total protein is 6.34% by weight.

The pyridine soluble extract prepared in accordance with the teachings of this invention has been found to have the following elemental analysis:

Element	Weight Percent
Carbon	60.35
Hydrogen	9.47
Oxygen	23.91

Additionally the extract is characterized by an infrared spectrum wherein the important peaks useful in identifying the extract are set forth in Table 1 below:

Peak Frequency* (cm^{-1})	Identification
3400(b)	NH Stretch
3200–2500(b)	Intramolecular hydrogen bonded OH peak
2920(s)	CH Stretch
1710(s)	Ester carbonyl Stretch
1675(s)	Amide carbonyl Stretch (Amide I Band)
1541(m)	Amide II Band

*(b) = broad
(s) = strong
(m) = moderate

Any microorganism may be used to obtain the pyridine-soluble extract including, for example, M.bovis BCG, *M.phlei, M.smegmatis, M.kansaii, Nocardia rubra, Corynebacterium diphtheriae* and *Corynebacterium parvum. Corynebacterium parvum* is especially preferred.

Whole cells of the microorganism, preferably in the form of a paste, are mixed with pyridine. The resulting mixture is separated to obtain a supernatant fraction which contains the pyridine-soluble extract and a pyridine residue. Optionally, the pyridine residue may be subjected to repeated separation procedures as described above using pyridine to remove further quantities of the desired extract.

The pyridine is then removed from the extract and the dried extract is dialyzed against a suitable liquid such as distilled water. The absence of whole cell and cell fragment contaminants is confirmed by electron microscopy. The resulting purified extract may then be lyophilized by known methods to obtain a stable project.

The immunological adjuvants of the present invention in admixture with the tumor associated antigens enhance the immune response against such antigens and hence are useful in a variety of vaccines tumors in for both animal and human hosts. In practice it has been found that the refined detoxified endotoxin (MPL) is used in a concentration of from about 10 to about 500 micrograms per dose with a particularly enhanced immune response being elicited at concentration of from about 10 to about 50 micrograms per dose. The cell wall skeleton is preferably used in concentration of from about 275 to about 325 micrograms per dose. The trehalose dimycolates are preferably used in a concentration of from about 50 to about 300 micrograms per dose, and more preferably from about 125 to about 175 micrograms per dose. The pyridine-soluble extract can be used in a concentration of from about 500 to about 2400 micrograms per dose and more preferably from about 750 to about 1200 micrograms per dose. If desired, other components or additives can be employed in conjunction with the adjuvants of the present inventions.

In those instances wherein only one or two of the three classes of adjuvants are employed with the antigen and MPL, the concentrations of such adjuvants may be adjusted to higher levels. All that is needed, however, is an immune response enhnacing amount of the adjuvant(s) which will enhance the immune response of the vaccine against the tumor associated antigen(s).

The optimum amount of antigen employed in the vaccines of the present invention will, of course, vary

for each particular tumor. In practice, however, it has been found that the antigen is generally present in the vaccine in a concentration of from about 1 to about 100 mg per dose, and more preferably, from about 2 to about 10 mg per dose.

The tumor associated antigens and the adjuvants are preferably employed in a pharmaceutically acceptable carrier to form the vaccine of this invention. Illustrative carriers which can be employed include, physiological saline or oil droplet emulsions. The amount of oil used is in the range of between about 0.5 and about 3.0 pecent by volume based on the total volume of the composition.

Preparation of the vaccines of this invention can be accomplished by blending the TAA, MPL and biological adjuvants in accordance with accepted techniques.

As described above the composition for treatment of warm blooded animals and humans may be used in the form of an oil droplet emulsion. The amount of oil used is in the range of between about 0.5 and 3.0 percent by volume based on the total volume of the composition. It is preferred to use between about 0.75 and 1.5 percent by volume of the oil. Examples of such oils include light mineral oil, squalane, 7-n-hexyloctadecane. Conoco superoil and Drakeol 6 VR mineral oil (produced by the Pennreco Company, Butler, Pa.).

The homogenized oil containing mixture is then combined with a detergent which may optionally be dissolved in a saline solution prior to mixing. The amount of detergent is typically between about 0.02 and 0.20 percent by volume and preferably between about 0.10 and 0.20 percent by volume based on the total volume of the composition. Any common detergent material may be used including Tween-80, and Arlacel (produced by the Atlas Chemical Company).

The mixture resulting from the addition of detergent is then homogenized to form a suspension which has a high percentage of oil droplets coated with MPL and CWS as determined by observation under a microscope.

Alternatively, aqueous suspensions of TAA may be added to lyophilized emulsions containing the biological adjucants and mixed or emulsified by vortexing until a slight milky suspension is obtained. Lyophilized emulsions are prepared as previously described (See U.S. Patent No. 4,520,019).

The vaccines of the present invention are usually administered to a warm blooded animal by intramuscular, intraperitoneal or subcutoneous injections once a week for up to a total of 15 injections in the doses indicated above.

It has been found that the vaccines of the present invention greatly enhance the immune response against a wide variety of natural tumor associated antigens; and both natural and synthetic viral, bacterial, fungal, or protozoan antigens. The only requirement of the antigen which is employed in the vaccines of the present invention is that it be capable of eliciting an immune response in a host and that the response will be enhanced by the adjuvants of this invention with which it is combined. Thus, the vaccines of the present invention have a potent anti-tumor activity and accordingly are useful in the treatment and prevention of a variety of tumors in both animals and humans. Tumors, including cancers, which may be treated by the vaccines include animal tumors such as bovine squamous cell carcinoma, bovine fibrosarcoma, equine sarcoid, equine melanoma, equine squamous cell carcinoma, canine mammary tumors, canine adenoma and canine melanoma, and human tumors such as ovarian cancers, melanomas, colorectal cancers, pancreatic cancers, renal cancer, and the like.

While not wishing to be bound by the mechanism as to how the vaccines of the present invention bring about tumor regression, it is believed that the combination of the tumor associated antigens and the adjuvant stimulate both a non-specific and specific immune response.

Although the vaccines of the present invention are effective in the treatment of tumors, in practice, these anti-tumor vaccines can be used in a clinical setting as a companion treatment to other forms of therapy. This is because immunotherapy can be most effective when the tumor burden is small enough that it can be handled by the patient's immune system. Thus, a patient with advanced disease would probably undergo some form of treatment to reduce the tumor burden, and subsequently would receive the anti-tumor vaccine in order to eliminate the residual tumor cells. The companion treatment may be surgery, chemotherapy, or radiation therapy, or any other method of effectively reducing the tumor burden. The companion treatment may even entail another form of immunotherapy, such as, for example, administration of interleukin-2.

In the examples which follow, the bacterial components, tumors and tumor cells, and tumor cell vaccines were prepared and evaluated employing procedures known in the art.

EXAMPLE 1

Preparation of Bacterial Components

1. Preparation of Detoxified endotoxin or monophosphoryl lipid A (MPL)

Crude endotoxin is isolated from the polysaccharide-deficient heptoseless Re mutant of *Salmonella minnesota* (strain R595) by organic solvent extraction. This strain was obtained from NIH, NIAID, Rocky Mountain Laboratory, Hamilton, Montana. This endotoxin, which consists only of KDO and lipid A, is a glycolipid rather than a typical endotoxic lipopolysaccharide and is purified by fractional precipitation with organic solvents of appropriate polarities. It is then treated with boiling 0.1 N hydrochloric acid to yield a complex mixture consisting of free fatty acids and structural homologs of non-toxic monophosphoryl lipid A (MPL). These components are separated by pressure elution column chromatography. Eluted fractions corresponding to structural homologs of MPL, as identified by thin-layer chromatography, are pooled and tested for toxicity. They qualify for experimentation in animals when their 50% lethal dose for intravenously inoculated chicken embryos ($CELD_{50}$) is greater than 10 μg. (The lethal dose for the parent endotoxin is less than 0.001 μg.)

2. Preparation of Mycobacterial Cell Wall Skeleton (CWS)

Cell walls of *Mycobacterium bovis*, strain BCG obtained from NIH, NIAID, Rocky Mountain Laboratories, Hamilton, Montana are prepared with the aid of the Sorvall-Ribi Cell Fractionator (Model RF-1). By using a pressure of 35,000 psi at a temperature of 10°-15° C., the mycobacterial cell walls are "cracked", and the protoplasm is extruded in a soluble state. Cell wall envelopes are then harvested by centrifugation and purified by repeated centrifugation and resuspension in

water. The cell walls are then treated with RNA-ase and DNA-ase to remove nucleic acids followed by a series of proteolytic enzymes and a detergent treatment to remove proteins and peptides, respectively. Finally the preparation is exhaustively extracted with organic solvents to remove "free lipid". The resulting CWS is composed of a polymeric mycolic acid-arabinogalactanmucopeptide complex.

3. Isolation and Purification of Trehalose-Dimycolate (TDM)

Whole cells of mycobacteria are extracted first with ethanol followed by acetone, and finally with a mixture of chloroform and methanol (CM 2:1). The CM extract contains the TDM plus contaminating lipids having lower or higher polarities than TDM. These lipids are selectively separated by precipitating them with compositions of organic solvents in which they are insoluble while retaining the TDM in a soluble phase. The resulting "crude TDM" is purified by pressure elution chromatography. Eluted fractions containing a single component of TDM as determined by TLC are pooled and used for study.

4. Pyridine Extraction of Corynebacterium Parvum (PE)

Heat-killed whole cells of *C. parvum VPI* 0204 obtained from Dr. C. Cummings, Virginia Polytechnic Institute, are extracted three times with pyridine at 37° C. and the combined pyridine soluble extracts are concentracted by flash evaporation, dialyzed and lyophilized. A substance with enhance anti-tumor activity and greatly reduced toxicity is obtained.

EXAMPLE 2

Preparation of Murine Tumor Models to be Evaluated

1. Animals

All tumor experiments ae performed on 6–8 week old C3HB/FeJ female mice or on C57BL/10 and DBA/2 mice of either sex. Mice are obtained from production colonies of Ribi ImmunoChem Research, Inc., Hamilton, Montana. Parental stocks of DBA/2 and C3HB/FeJ mice are purchased from the Jackson Laboratory (Bar Harbor, Maine).

Sewell-Wright guinea-pigs of inbred strain-2 are obtained from the production colony of Ribi ImmunoChem Research. Guinea-pigs of either sex will weigh 350–500g on entry in the experiments.

2. Tumors

Leukemia EL-4 (Provided by Dr.Bruce Chesebro NIAID, Rocky Mountain Laboratory, Hamilton, Montana), ovarian MOT (from Dr. J. Berek, UCLA, Los Angeles, Ca.), and lymphoma P388 cells obtained from ATCC are maintained in the ascitic form by serial transfers in C57BL/10, C3HB/FeJ, and DBA/2 mice, respectively. Tumor cells are harvested from the peritoneal cavity and washed with 20 ml phosphate buffered saline (PBS). Red blood cells (RBC) are lysed with 10 ml of 1% ammonium oxalate. After 30 sec., physiological osmolarity is restored by the addition of 40 ml PBS. Cells are pelleted by centrifugation, and the cell dose is adjusted so that an inoculum of 2 to 5×10^4 cells was administered in 0.1 ml of PBS. Line -10 hepatoma cells are maintained in ascitic form in syngeneic strain 2 guinea-pigs by serial i. p. transfer.

Tumor cells are harvested from ascites-bearing donors, washed three 3 times in sterile saline, and adjusted to 20×10^6 tumor cells/ml.

3. Design of Immunotherapy Experiment

EL-4, MOT, or P388 tumor cells obtained from the same source as indicated above are prepared as described above and 0.2 ml of tumor cell suspension is inoculated i. p. in the appropriate syngeneic strain of mice on day 0. Tumor controls receive no further treatment. At various times (day 1 to day 6) after tumor transplantation, mice are given a single i. p. injection of the immunotherapeutic. Animals in each group are observed daily to determine percentage survival and/or reduction in tumor mass. The line-10 tumor is established in guinea-pigs by intradermal injection of 10^6 ascites-grown tumor cells. The immunotherapeutics are administered in 0.4 ml volumes by intralesional inoculation after 6 days, at which time the tumors are 9–11 mm in diameter.

EXAMPLE 3

Formulation Procedures for Preparation of Tumor Cell Vaccines

1. Preparation of Solubilized Tumor Associated Antigen(s)

Tumor antigen is prepared by a modified procedure using 3 M KCl extraction. Briefly, 3 M KCl in PBS is added to a cell pellet of live tumor cells at a concentration of 5 $m\frac{1}{2}\times10^8$ cells. The suspension is stirred for 18 to 24 hours at 4°, after which the pellets are discarded and the supernatant fluid is dialyzed against distilled water for 24 hours at 4° and then centrifuged at 100,000 x g for 2 hours at 4°. The dialysate is finally centrifuged at 100,000 x g for 2 hours at 4° to remove the fine gelatinous precipitate that forms after dialysis. The soluble material is then lyophilized.

The Line-10 guinea-pig tumor; murine E1-4, MOT, and P388; as well as bovine ocular squamous cell carcinoma (BOSCC) and equine sarcoid (ES) obtained from tumor bearing hosts were extracted extracted in this manner. In addition saline suspensions of homogenized BOSCC and ES are extracted by a modified phenol procedure.

2. Preparation of Oil Droplet Emulsion

Oil droplet emulsions contained soluble extracts of the tumors are parepared with varying combinations of CWS, TDM, and MPL as described previously (U.S. Pat. No. 4,520,019).

3. Preliminary Testing of Tumor Vaccines

The methods of therapy are essentially as described previously. Basically, tumors are established by s. c. injection of mice or guinea-pigs with 2 to 5×10^4 ascites grown tumor cells. Twenty five or 100 μg quantities of mitomycin C are administered intralesionally 7 days after the implantation, when the tumors are about 4 mm in diameter. Two days later, the vaccines are injected by various routes. Cure rates are compared with the survival of untreated animals. Cured animals are tested for capacity to resist a subsequent challenge with homologous tumor cells.

4. Specific Immunotherapy of Spontaneous Tumors of Cattle and Horses

Bovine ocular squamous cell carcinoma (BOSCC) is a potentially metastatic autochthonous carcinoma occurring naturally in about 4.7% of Hereford cattle and in 0.8% - 1.6% of the general cattle population. Equine sarcoid (ES) is a locally aggressive skin tumor and is the most common spontaneous tumor of horses. These tumors do not metastasize readily, but are locally invasive and are nonaggressive.

EXAMPLE 4

Isolation of Tumor Associated Antigens

Two experimental tumor models, (a) line-10 hepatocellular carcinoma in inbred strain 2 guinea-pigs and (b) Murine ovarian tumor (MOT) in inbred C3HB/FeJ mice were obtained from existing breeding colonies of these recipient animals at Ribi ImmunoChem Research, Inc., Hamilton, Montana.

Three following additional murine tumor of cell lines were obtained: L-1210 leukemia was obtained from Dr. Jerry Killion, Oral Roberts University, Tulsa, OK; EL-4 lymphoma tumor cells were obtained from Dr. Bruce Chesebro, NIAID, Rocky Mountain Laboratory, Hamilton, Montana; and P-388 lymphoma tumor cells were purchased from the ATCC. $B_6D_2F_1$ and C3HB/FeJ mice for the maintenance of these tumor cell lines and for tumor regression studies were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Tumor associated antigens (TAA) for use in combination with MPL and other bacterial anti-tumor components of this invention were isolated from MOT ovarian tumor cells, EL-4 lymphoma, P-388 lymphoma and L-1210 leukemia by the 3M KCl extraction method. Briefly, groups of the appropriate strain of mice were given as i. p. injection of 0.5 to 1×10^5 viable tumor cells. Ascites fluid was collected 8 to 10 days later and cells removed by centrifugation. Marked tumor cells were extracted overnight with 3M KCl. The aqueous soluble extract was obtained by centrifugation, dialyzed and lyophilized. In addition, TAA was also obtained from ascites grown line-10 tumor cells in strain 2 guinea-pigs using the above procedure.

TAA extracts were also obtained from two spontaneous arising sarcoid tumors in horses. The solid sarcoid tumors were homogenized in 3M KCl and extracted at 4° C. overnight. The aqueous extract was collected by centrifugation, dialyzed and lyophilized.

EXAMPLE 5

Anti-tumor Activity of Detoxified Endotoxin (MPL) Alone or

Combined with Pyridine Extract of *P. acnes* (PA-PE)

Experiments were conducted to show that PA-PE and mycobacteria trehalose dimycolate (TDM) were effective as cell wall skeleton (CWS) in regressing line-10 tumors in strain 2 guinea-pigs when combined with MPL. The results obtained are set forth in Table 1 below:

TABLE 1

Anti-tumor Activity of Oil Emulsions Containing MPL Alone or Combined with PA-PE + TDM in the Line-10 Tumor Model[a]

Material Injected	Dose	Number Cured Total	% Cured
PE *C. parvum* + MPL + TDM	500 + 50 + 100	17/19	89.5
PE *C. parvum* + MPL + TDM	100 + 50 + 50	5/10	50
PE *C. parvum* + TDM	500 + 100	3/9	33
PE *C. parvum* + MPL	500 + 50	1/9	11
PE *C. parvum* only	500	0/7	0
MPL + TDM	500 + 100	0/6	0
Tumor Controls	—	0/18	0

[a]Strain 2 guinea-pigs were inoculated I.D. with 2×10^4 line-10 tumor cells on day 0. Immunostimulants were administered intralesionally (0.4 ml/animal) on day 9 when the tumors were 8 to 10 mm in diameter.

EXAMPLE 6

The following studies were designed to determine the ability of the aqueous solutions containing MPL alone or in combination with PA-PE to regress MOT ovarian cells in C3HB/FeJ mice. Female mice were inoculated with 2×10^4 MOT cells on day 0 and immunotherapy was administered 24 hours later. As shown in Table 2, little or no anti-tumor activity was observed in mice given either 1200 μg of PA-PE or 240 μg or MPL. However, signifigant anti-tumor activity (88% tumor free) was seen in mice treated with the combination of PA-PE +MPL. Moreover, the response was dose dependent in that fewer tumor free animals were seen with decreasing doses of immunostimulants. The results are shown in Table 2 below:

TABLE 2

Anti-tumor Activity of Detoxified Endotoxin (MPL) Alone or Combined with Pyridine Extract of *P. acnes* (PA-PE)[a]

Material Injected	Dosage (μg)	No. Tumor Free[b] Total No. Injected	Percent Tumor Free
PA-PE + MPL	1200 + 240	43/49	88
PA-PE + MPL	600 + 120	4/8	50
PA-PE + MPL	350 + 75	0/5	0
MPL	240	2/8	25
PA-PE	1200	0/8	0
None	—	0/54	0

[a]Female C3HB/FeJ mice inoculated i.p. with 1-2 × 10^4 MOT tumor cells on day 0. Immunostimulants were administered i.p. (0.5 ml/mouse) 24 hours following tumor transplantation.
[b]Number tumor free was determined 60 days after tumor transplantation.

EXAMPLE 7

To determine whether solubilized tumor antigens (TAA) would enhance or add to the antitumor activity of PA-PE +MPL, mice-bearing MOT tumors were given MOT-TAA alone or in combination with a non efficacious dose of PA-PE +MPL (Table 3). No anti-tumor activity was observed in mice treated with 500 or 350 μg of TAA only. However, when 500 μg of TAA was combined with PA-PE +MPL. 1005 of the mice were tumor-free 60 days after tumor transplantation. The mean survival time for the nontreated tumor control group was 21 days. The results are set forth in Table 3 below:

TABLE 3

Efficacy of Tumor Associated Antigens (TAA) Alone or in Combination with PA-PE + MPL in Regressing MOT Tumors in C3HB/FeJ Mice[a]

Material Injected	Dosage (μg)	No. of Tumor Free[b] Total No. Injected	Percent Tumor Free
TAA	500	0/8	0
TAA	350	0/5	0
PA-PE +MPL	350 + 75	0/5	0
TAA + PA-PE + MPL	350 + 350 + 75	2/5	40

TABLE 3-continued

Efficacy of Tumor Associated Antigens (TAA) Alone or in Combination with PA-PE + MPL in Regressing MOT Tumors in CJHB/FeJ Mice[a]

Material Injected	Dosage (μg)	No. of Tumor Free[b] Total No. Injected	Percent Tumor Free
TAA + PA-PE + MPL	500 + 300 + 60	8/8	100

[a]Female CJHB/FeJ mice were inoculated i.p. with 1-2 × 10^4 MOT tumor cells on day 0. Immunostimulants were administered i.p. (0.5 ml/mouse) 24 hours following tumor transplantation.
[b]Number tumor free was determined 60 days after tumor transplantation.

EXAMPLE 8

Although significant anti-tumor activity was observed with TAA combined with PA-PE +MPL, it was of interest to determine its anti-tumor effect when administered at increasing times post tumor transplantation. Mice were given 2×10^4 MOT cells on day 0 and therapy (PA-PE +MPL or TAA +PA-PE +MPL) on day 1,2,3 or 4. Effective therapy with PA-PE +MPL alone or in combination with TAA was observed when given on day 1 or 2 post tumor cell inoculation. However on days 3 and 4, the amount of anti-tumor activity as measured by tumor free animals was significantly reduced. This decrease in anti-tumor activity is likely due to an increase in tumor burden. Therefore, studies were designed to reduce tumor burden with chemotherapy using mitomycin C. The results are summarized in Table 4 below:

TABLE 4

Anti-tumor Activity of MPL + PA-PE or TAA + MPL + PA-PE Given at Various Times After Tumor Transplantation.[a]

Material Injected	Time After Tumor (days)	No. Tumor Free[b] Total No. Injected	Percent Tumor Free
Pa-PE + MPL	1	5/6	83
(1200 μg + 240 μg)	2	4/6	67
	3	1/6	17
	4	0/6	0
TAA + PA-PE + MPL	1	5/6	83
(500 + 300 + 60)	2	4/6	67
	3	1/6	17
	4	0/6	0
TAA only (500)	1	0/6	0
None	—	0/6	0

[a]Female CJHB/FeJ mice inoculated i.p. with 1-2 × 10^4 MOT tumor cells on day 0. Immunostimulants were administered i.p. (0.5 ml/mouse) at 24 hour intervals following tumor transplantation.
[b]Number tumor free was determined 60 days after tumor transplantation.

EXAMPLE 9

To test the effect of combination chemoimmunotherapy, mice were given MOT tumor cells on day 0 and i.p. mitomycin C on days 2 to 6. Immunotherapy was administered 30 to 36 hours after drug administration. The dosage of mitomycin C used was predetermined by treating tumor bearing mice on day 6 with varying dosages of drug i.p.. A dose of 100 μg was selected based on its lack of toxicity and minimal therapeutic effect (20% regression rate).

Table 5 shows the results of a study where tumor bearing mice were treated with mitomycin C on day 6 and immunotherapy 31 hours later. All mice treated with immunotherapy without prior chemotherapy died of progressive tumor growth (data not shown). However, a 50% response rate was observed in the group receiving drug and TAA +PA-PE +MPL at high dose with minimal activity seen at the lower dosage.

TABLE 5

Effect of Chemotherapy Followed by PA-PE + MPL + MOT – TAA on MOT in CJHB/FeJ Mice[a]

Material Injected; 31 h post Chemotherapy	dose (μg)	No. in Regression total injected Day 34	Day 63	% Responding Day 34	Day 63
TAA	500	0/6		0	0
TAA + PA-PE + MPL	500 + 300 + 60	3/6	1/6	50	17
TAA + PA-PE + MPL	500 + 1200 + 240	3/6	3/6	50	50
PA-PE + MPL	300 + 60	0/6		0	0
PA-PE + MPL	1200 + 240	2/6	1/6	33	17
MOT cells only, d0	—	0/6		0	0

[a]Female CJHB/FeJ mice were inoculated i.p. with 1-2 × 10^4 MOT tumor cells on day 0. Chemotherapy was given i.p. on day 6 followed by immunotherapy 31 hours later.

EXAMPLE 10

The procedure of Example 9 was repeated with the exception that the time between tumor transplantation and chemotherapy was varied. Immunotherapy was given 33 hours after the drug mitomycin. The results are shown in Table 6 below:

TABLE 6

Effect of Chemotherapy on Days 2,4, or 6 Followed by PA-PE + MPL + MOT – TAA, on MOT in CJHB/FeJ Mice[a]

Group	Material Injected 33 h post.chemotherapy	dose (μg)	No responding d34 Total injected	% responding	MST
A	PA-PE + MPL	300 + 60	4/6	67	>34
B	PA-PE + MPL	1200 + 240	5/6	83	"
C	TAA + PA-PE + MPL	500 + 300 + 60	5/6	83	"
D	"	500 + 1200 + 240	6/6	100	"
E	"	1000 + 300 + 60	4/6	67	"
F	"	1000 + 1200 + 240	5/6	83	"
G	Control, MOT cells only	—	4/6	67	"
H	PA-PE + MPL	1200 + 240	5/6	83	>34
I	TAA + PA-PE + MPL	500 + 300 + 60	5/6	83	"
J	"	500 + 1200 + 240	6/6	100	"
K	"	1000 + 300 + 60	4/6	67	"

TABLE 6-continued

Effect of Chemotherapy on Days 2,4, or 6 Followed by PA-PE + MPL + MOT – TAA, on MOT in C3HB/FeJ Mice[a]

Group	Material Injected 33 h post chemotherapy	dose (μg)	No responding d54 Total injected	% responding	MST
L	"	1000 + 1200 + 240	5/6	83	"
M	Control, MOT cells only	—	0/6	0	28
N	TAA	1000	1/6	17	35
O	TAA + PA-PE + MPL	500 + 1200 + 240	3/6	50	37
P	"	1000 + 300 + 60	3/6	50	43
Q	"	1000 + 1200 + 240	4/6	67	>54
R	Control, MOT cells only	—	0/6	0	24
S	Mot cells only (no drug)	—	0/6		22

[a]Groups A–O, mitomycin day 2;
Groups H–M, mitomycin day 4;
Groups N–R, mitomycin day 6.

When mitomycin C was administered on day 2, no signifigant difference was observed in those groups receiving chemoimmunotherapy or chemotherapy only. Thus, chemotherapy has a dramatic effect when given early. Conversely no chemotherapeutic effect was seen in animals given the drug only on day 4 or 6 but when combined with immunotherapy, a marked anti-tumor effect was observed. This anti-tumor activity was seen in mice given PA-PE +MPL alone or combined with TAA.

EXAMPLE 11

Anti-tumor Activity of TAA in combination with Other Microbial Anti-tumor Components in Regressing line-10 Tumors in Strain 2 Guinea-Pigs

Previous studies have shown that the combination of MPL and cell wall skeleton (CWS) has signifigant anti-tumor activity as measured by regression of established line-10 tumors in strain 2 guinea-pigs. The following study was designed to determine whether the addition of solubilized TAA to the MPL +CWS would enhance its anti-tumor activity. Inbred strain 2 guinea-pigs were inoculated with 2×10^6 viable line-10 tumor cells on day 0. Immunotherapy was given directly into the tumors as oil-in-water emulsions on day 6 when tumors were 8 to 10 mm in diameter. Results are shown on Table 7.

TABLE 7

Anti-tumor Activity of Tumor Associated Antigen in Combination with Various BRM Formulations on Line-10 Hepatocarcinoma in Strain 2 Guinea Pigs

Material Injected	dose (μg)	No. Tumor Free total injected	% Tumor Free (day 84)
Triple mixture (CWS + MPL + TDM)	50 + 50 + 50	3/7	43
L10-TAA + Triple mixture	1000 50 + 50 + 50	5/7	71
DETOX (CWS + MPL)	200 + 20	1/7	14
L10-TAA + DETOX	1000 200 + 20	2/7	29
L10-TAA only	1000	0/7	0

[1]Results shown are for intratumor injection. No anti-tumor activity was observed (0/7) when these combination were given in contralateral flank.

Although the invention has been illustrated by the preceding examples, it is not to be construed as being limited to the materials disclosed therein; but rather the invention is directed to the generic area as hereinbefore disclosed. Various modification an embodiments can be made without departing from the spirit or scope thereof.

What is claimed is:

1. A vaccine useful for the treatment and prevention of tumors in a host, said vaccine comprised of:
(a) at least one tumor-associated antigen,
(b) a refined detoxified endotoxin immunostimulant, and
(c) at least one biological immunostimulant selected from the group consisting of:
(1) mycobacterial cell wall skeleton
(2) trehalose dimycolate, and
(3) pyridine soluble extract of a microorganism, and
(d) a pharmaceutically acceptable carrier.

2. The vaccine of claim 1 wherein said refined detoxified endotoxin has no detectable 2-keto-3-deoxyoctonoate between about 350 and 475 nmoles/mg of phosphorus and between about 1700 and 2000 nmoles/mg of fatty acids.

3. The vaccine of claim 1 wherein said refined detoxified endotoxin is monophosphoryl lipid A.

4. The vaccine of claim 1 wherein component (c) is mycobacterial cell wall skeleton.

5. The vaccine of claim 1 wherein component (c) is trehalose dimycolate.

6. The vaccine of claim 1 wherein component (c) is a pyridine soluble extract of a microorganism.

7. The vaccine of claim 1 wherein component (c) is a combination of mycobacterial cell wall skeleton and trehalose dimycolate.

8. The vaccine of claim 1 wherein component (c) is a combination of mycobacterial cell wall skeleton and a pyridine soluble extract of a microorganism.

9. The vaccine of claim 1 wherein component (c) is a combination of trehalose dimycolate and a pyridine soluble extract of a microorganism.

10. The vaccine of claim 1 wherein component (c) is a combination of mycobacterial cell wall skeleton, trehalose dimycolate and a pyridine soluble extract of a microorganism.

11. The vaccine of claim 1 wherein said pyridine soluble extract from said microorganism contains between about 7 and 20% by weight of protein, between about 10 and 16% by weight of sugar, and between about 35 and 55% by weight of fatty acids.

12. A process for the preparation fo a vaccine useful for the treatment and prevention of tumors in a host which comprises forming a mixture of:
(a) at least one tumor associated antigen
(b) a refined detoxified endotoxin
(c) at least one biological immunostimulant selected from the group consisting of:
(1) mycobacterial cell wall skeleton
(2) trehalose dimycolate, and
(3) pyridine soluble extract of a microorganism, and

(d) a pharmaceutically acceptable carrier

13. A process for the treatment and prevention of tumors in a host, which comprises administering to said host an anti-tumor effective amount of a vaccine comprised of:

(a) at least one tumor associated antigen,

(b) a refined detoxified endotoxin immunostimulant, and

(c) at least one biological immunostimulant from the group consisting of:

(1) mycobacterial cell wall skeleton,

(2) trehalose dimycolate, and

(3) pyridine soluble extract of a microorganism, and

(d) a pharmaceutically acceptable carrier.

14. The process of claim 13 wherein said refined detoxified endotoxin has no detectable 2-keto-3-deoxyoctonoate, between about 350 and 475 nmoles/mg of phosphorus and between about 1700 and 2000 nmoles/mg of fatty acids.

15. The process of claim 13 wherein said refined detoxified endotoxin is monophosphoryl lipid A.

16. The process of claim 13 wherein component (c) is mycobacterial cell wall skeleton.

17. The process of claim 13 wherein component (c) is trehalose dimycolate.

18. The process of claim 13 wherein component (c) is a pyridine soluble extract of a microorganism.

19. The process of claim 13 wherein component (c) is a combination of mycobacterial cell wall skeleton and trehalose dimycolate.

20. The process of claim 13 wherein component (c) is a combination of mycobacterial cell wall skeleton and a pyridine extract of a microorganism.

21. The process of claim 13 wherein component (c) is a combination of trehalose dimycolate and a pyridine soluble extract of a microorganism.

22. The process of claim 13 wherein component (c) is a combination of mycobacterial cell wall skeleton, trehalose dimycolate and a pyridine soluble extract of a microorganism.

* * * * *

EXHIBIT 12

B

United States Patent [19]

Myers et al.

[11] Patent Number: 4,912,094

[45] Date of Patent: Mar. 27, 1990

[54] MODIFIED LIPOPOLYSACCHARIDES AND PROCESS OF PREPARATION

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[73] Assignee: Ribi ImmunoChem Research, Inc., Hamilton, Mont.

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[22] Filed: Jun. 29, 1988

[51] Int. Cl.[4] C07H 1/00; C07H 13/02; C07G 17/00; C12P 19/04

[52] U.S. Cl. 514/54; 536/124; 536/1.1; 536/119; 536/117; 536/115; 435/101

[58] Field of Search 536/124, 1.1, 119, 115, 536/117; 435/101; 514/54

[56] References Cited

U.S. PATENT DOCUMENTS

3,089,821	5/1963	Folkers	514/54
4,029,762	6/1977	Galanos et al.	530/387
4,185,090	1/1980	McIntire	536/1.1

Primary Examiner—Ronald W. Griffin
Assistant Examiner—Everett White
Attorney, Agent, or Firm—Burgess, Ryan and Wayne

[57] ABSTRACT

Modified lipopolysaccharides, particularly de-3-O-acylated monophosphoryl lipid A and de-3-O-acylated diphosphoryl lipid A, are provided by an alkaline hydrolysis under controlled conditions which removes only the β-hydroxymyristic acyl residue that is ester-linked to the reducing-end glucosamine at position 3. The modified products are less endotoxic and maintain their antigenic and immuno-stimulating properties.

26 Claims, No Drawings

MODIFIED LIPOPOLYSACCHARIDES AND PROCESS OF PREPARATION

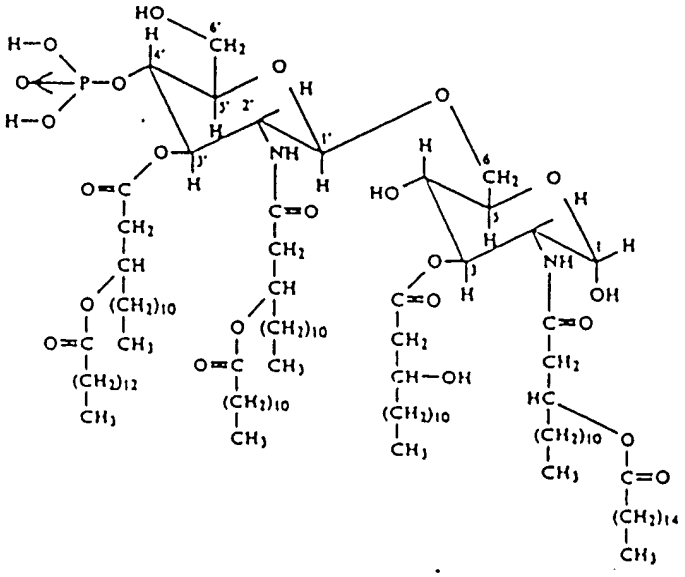
FIELD OF THE INVENTION

This invention relates in general to certain modified forms of lipopolysaccharide and lipid A. In one aspect, this invention is directed to a process for the structural modification of certain lipopolysaccharides to render them less endotoxic without adversely affecting their antigenic or immuno-stimulating properties.

BACKGROUND OF THE INVENTION

Prior to the present invention, it had long been recognized that enterobacterial lipopolysaccharides (LPS) was a highly potent stimulator of the immune system. A variety of responses, both beneficial and harmful, can be elicited by sub-microgram amounts of this substance. The fact that some of these responses are harmful, and can in fact be fatal, has to date precluded clinical use of LPS per se. It is now also well-appreciated that the endotoxic activites associated with bacterial lipopolysaccharides (LPS) reside in the lipid A component of LPS.

Accordingly, much effort has been expended towards attenuating the toxic attributes of lipid A and LPS without diminishing their beneficial immunostimulatory activities. Notable among these efforts was that of Edgar Ribi and his associates, which resulted in the production of a derivative of lipid A referred to originally as refined detoxified endotoxin (RDE) but more recently as monophosphoryl lipid A (MPL). MPL is produced by refluxing LPS (or lipid A) obtained from heptoseless mutants of gram negative bacteria (e.g. Salmonella sp.) in mineral acid solutions of moderate strength (e.g., 0.1N HCl) for a period of approximately 30 minutes. This treatment results in the loss of the phosphate moiety at position 1 of the reducing-end glucosamine. Coincidentally, the core carbohydrate is removed from the 6' position of the non-reducing glucosamine during this treatment. The result is the monophosphoryl derivative of lipid A, MPL. The structure of MPL is shown below:



(I)

MPL exhibits considerably attenuated levels of the endotoxic activities normally associated with lipid A and LPS, such as pyrogenicity, local Shwartzman reactivity, and toxicity in the chick embryo 50% lethal dose assay ($CELD_{50}$). It retains the ability of lipid A and LPS, however, and to, among other things, act as an adjuvant.

The difficulty with this method of detoxifying LPS and lipid A is that it invariably results in the loss of the core moiety attached to position 6' of the non-reducing glucosamine. This is significant since the core region is highly conserved among LPS's obtained from different genera of Enterobacteriaciae; immunity against the core region is therefore protective against a wide variety of gram negative bacterial challenges. This was demonstrated by the work of Ziegler et al. (New Eng. J. Med. 307, 1225: 1982), for example.

Considerable benefits would accrue from being able to immunize individuals against enterobacterial LPS, as evidenced by the fact that approximately 90,000 deaths occur annually from gram negative sepsis and associated endotoxemia. At the present time, however, it is only possible to immunize with fully toxid LPS, since detoxification by acid hydrolysis results in loss of the core region.

Alkaline hydrolysis has also been used in the past to detoxify LPS, but the conditions which have generally been used result in complete saponification of the lipid A moiety. This, of course, not only reduces the endotoxicity of the starting LPS, but also eliminates the other, more beneficial, activities as well. Furthermore, such treatment also reduces the immunogenicity of LPS, since it is essentially converted by this treatment into a polysaccharide antigen with no amphipathic character. In general, however, none of the early references teach that removal of one particular fatty acid from lipid A would render it non-toxic, while not affecting its immunostimulating activities.

Accordingly, one or more of the following objects will be achieved by the practice of this invention. It is an object of this invention to provide modified lipopolysaccharrides and, in particular, modified lipid A. Another object of this invention is to provide a modified lipid A which retains the core moiety attached to the 6' positions of non-reducing glucosamine. A further object

of the present invention is to provide a modified lipid A which retains the core moiety and accordingly, protection against a wide variety of gram negative challenges. A still further object of the present invention is to provide a process for the preparation of the modified lipid A which renders it less endotoxic without adversely affecting their antigenic or immunostimulating properties. Another object is to provide pharmaceutical compositions containing the modified lipopolysaccharides and a method for their use. These and other objects will readily become apparent to those skilled in the art in light of the teachings herein set forth.

SUMMARY OF THE INVENTION

In its broad aspect, the present invention is directed to certain modified forms of lipopolysaccharide and lipid A, and to a process for their preparation. The invention also encompasses pharmaceutical compositions containing the modified lipopolysaccharides and their use in the treatment of various conditions in warm blooded animals.

The modified lipopolysaccharides and lipid A of this invention are those which have been subjected to a mild alkaline hydrolysis under conditions as hereinafter defined that result in the loss of a single fatty acid from position 3 of the lipid A backbone.

DETAILED DESCRIPTION OF THE INVENTION

There is a considerable body of literature which pertains to the effect of alkaline treatment on the biological activities of LPS and lipid A. Most of these references teach the use of conditions that are sufficient to completely deacylate lipid A. As stated earlier, such treatment destroys essentially all biological activity of lipid A and LPS, except for antigenicity. The early paper by Neter et al. (Neter E., Westphal O., Luderitz O., Gorzynski E. A. and Eichenberger E., "Studies of enterobacterial lipopolysaccharides". Effects of heat and chemicals on erythrocyte modifying, antigenic, toxic, and pyrogenic properties", J. Immunol. 76, 377: 1956), can be regarded as representative of the state of the art which teach the use of alkaline conditions sufficient to destroy all biological activities of LPS.

Several other observations have been noted in the scientific literature concerning alkaline hydrolysis of lipid A and LPS. For example, Niwa et al. (J. Bacterial. 97, 1069: 1969;) observed that treatment of LPS with mildly alkaline conditions caused a rapid loss of endotoxic activity and a much slower loss of fatty acids. This observation led them to conclude that the fatty acid-containing portion of LPS, lipid A, was not responsible for the endotoxic activity of LPS, since it was evidently destroyed at a slower rate than the endotoxic activity was lost. The authors conjectured that the only way that their observations might be consistent with lipid A being the endotoxic principle was if there existed a fatty acid in lipid A that was both highly alkaline-labile and necessary for endotoxic activity. The authors did not consider this to be a likely explanation. At the time, Niwa et al. favored an explanation for their results based on the influence of mild alkaline treatment on the conformation of endotoxin aggregates.

In a paper by Rietschel et al. (Eur. J. Biochem. 28, 166; 1973), it was noted that β-hydroxymyristic acid is rapidly released from lipid A upon mild alkali treatment (0.25N NaOH, 56° C.). The reason for the rapid loss of β-hydroxymyristic acid was not given, nor was it known from which position this fatty acid was cleaved. Also, no mention was made of the relationship of this rapid loss of β-hydroxymyristic acid to the loss of endotoxicity upon mild alkaline treatment which was observed by Niwa et al. and others.

In a paper by Goodman and Sultzer (Infect. Immunity 17, 205: 1977;) the authors noted that mild alkaline hydrolysis of LPS reduced its toxicity while actually enhancing its mitogenicity. They chemically characterized the hydrolyzed product with respect to nitrogen, glucosamine, KDO, and fatty acid content. Significantly, they found that the fatty acid content was relatively unchanged by the alkaline treatment. This led Goodman and Sultzer to conclude that the effect of the mild alkaline treatment was mediated by changes in the aggregational properties of the hydrolyzed LPS. In this regard, they were adopting the view of Niwa et al. On p. 212 of their paper, Goodman and Sultzer state that " . . . we have reduced the toxicity of the [LPS] by about 100-fold without significantly changing the lipid moiety." This confirms that they did not understand what they had done to achieve the observed reduction in toxicity without reducing mitogenicity. No mention was made of the possibility of a critical fatty acid that is alkaline-labile.

The sensitivity of ester-linked β-hydroxymyristic fatty acid residues present in lipid A to alkaline hydrolysis was noted in a 1982 publication (N. Qureshi, D. Takayama, and E. Ribi, J. Biol. Chem. 257, 11808: 1982). Similar observations were made with respect to a monosaccharide precursor of lipid A in a 1983 publication (Takayama, et al., J. Biol. Chem 258, 14245: 1983). Both of these references teach that ester-linked β-hydroxymyristic fatty acid residues present in lipid A or related compounds are easily removed by mild alkaline treatment. The effect of this structural modification on the biological activity of lipid A was not recognized in this or any subsequent references.

The treatment of LPS with mild alkali was discussed in two papers by Amano, et al. (D. Amano, E. Ribi, and J. L. Cantrell, J. Biochem 93, 1391: 1983, and K. Amano, E. Ribi, and J. L. Cantrell, BBRC 106, 677: 1982). The authors reported that mild alkali treatment results in the loss of O-ester linked fatty acids. They did not mention that the only fatty acid removed by this treatment is the β-hydroxymyristic at position 3. Also, contrary to the results disclosed in the present invention, they reported that mild alkali treatment did not reduce the endotoxicity of the parent LPS.

A study of the structural consequences of treating LPS with mild alkali was reported in a paper by Rosner, et al. (M. R. Rosner, J-y Tang, I. Barzilay, and H. G. Khorana, J. Biol. Chem 254, 5906: 1979). The authors reported that LPS which was treated with 1N NaOH at room temperature for approximately 17 hrs was exhaustively de-O-acylated. This is clearly different from the present invention, which discloses conditions sufficient to remove only the β-hydroxymyristic from position 3. Furthermore, the authors subjected LPS to this mild alkali treatment solely for the purpose of elucidating LPS's structure. No mention is made in this article of the effect of mild alkali treatment on the biological activities of LPS.

The use of mild alkali treatment to lower the toxicity of lipid A was disclosed in U.S. Pat. No. 4,029,762, this patent discloses the use of lipid A and alkali-treated lipid A as antigens for stimulating immunity against gram-negative enterobacteriaceae. It was not disclosed

in this patent that lipid A, which lacked a β-hydroxymyristic acid at position 3, is less endotoxic but is still mitogenic.

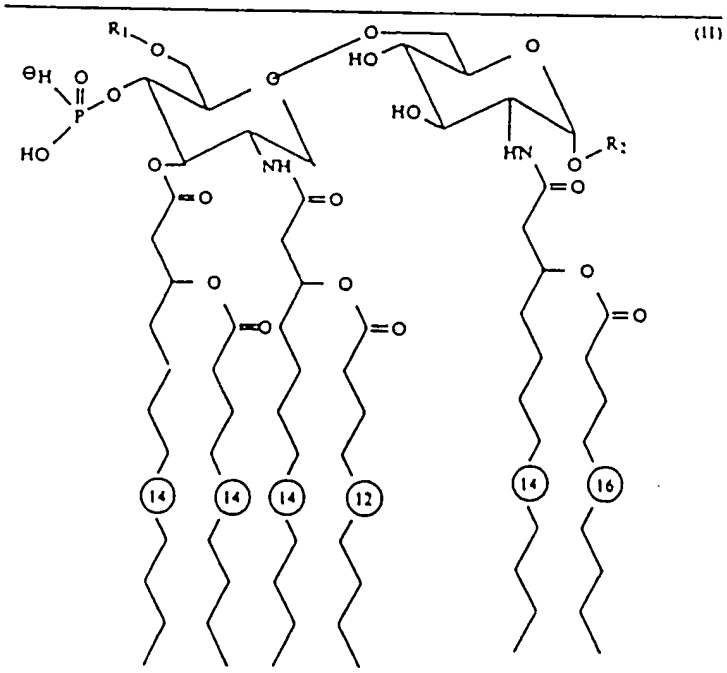
In a 1987 review by Rietschel et al. (in "Detection of Bacterial Endotoxins with the Limulus Amebocyte Lysate Test", Alan R. Liss, Inc., 1987, p. 25-53;), mention is made of the fact that synthetic monosaccharides corresponding to the reducing end of lipid A are inactive if the β-hydroxymyristic acid residue at position 3 is removed. The authors, however, did not conjecture as to whether the same observation would be made with lipid A, and no work was cited pertaining to this question. It does not appear then that it was suspected that removal of the β-hydroxymyristic acid from position 3 of lipid A and LPS would result in reduced endotoxicity without affecting activities such as mitogenicity.

Accordingly, prior to the present invention, and in view of the reported research efforts of Edgar Ribi and his colleagues, in preparing and evaluating monophosphoryl lipid A, it was generally recognized that detoxification of lipopolysaccharide was best accomplished by an acid hydrolysis followed by a chromatographic separation of MPL if a product having enhanced immuno-stimulating properties was desired. It was not readily apparent that the endotoxicity of lipid A could be attenuated by removal of only the fatty acid at position 3 or that removal of the position 3 fatty acid from lipopolysaccharide would reduce endotoxicity and yet allow other desirable substituents to remain in the molecule.

Thus, in contrast to the prior art references which may disclose, in general, the alkaline treatment of lipopolysaccharides, none of these references clearly recognizes the unexpected and surprising results obtained by employing conditions for removal of only the β-hydroxymyristic acyl residue from lipopolysaccharides and thereafter separating and recovering the deacylated product in a relatively pure form.

Lipid A deacylated in accordance with the method of the present invention was found to be non-toxic in the $CELD_{50}$ assay ($CELD_{50} > 10$ μg), in spite of the fact that it still contained (1) a diglucosamine backbone, (2) two phosphoryl groups, (3) at least two 3-acyloxyacyl residues, and (4) up to a total of 6 fatty acids. Taken together, these results indicate that the total number of fatty acids present in lipid A is not a sufficient condition for the manifestation of endotoxic activity, but that the pattern of fatty acid substitution is also a critical determinant.

While not wishing to be bound by any theory regarding the reasons why the compound(s), although less endotoxic, are still able to exert a strong immunostimulating effect, it is believed that the specific structural modification that is responsible for this reduction in the endotoxicity of lipid A and LPS involves removing of the β-hydroxymyristic acyl residue that is ester-linked to the reducing-end glucosamine at position 3 under conditions which do not adversely affect other groups in the lipopolysaccharides. Monophosphoryl lipid A (MPL), diphosphoryl lipid A (DPL) and LPS can all be de-3-O-acylated in this way. The structures of these novel materials are shown below in formula II wherein the figures in the circles indicates the number of carbon atoms in the chain.



(II)

Compound	R1	R2
dJ-MPL	H	H
dJ-DPL	H	PO_3H^-
dJ-LPS	Core (±O-antigen)	PO_3H^-

Various forms of de-3-O-acylated materials are encompassed by this invention. The lipid A backbone that is shown corresponds to the product that is obtained by de-3-O-acylation of heptaacyl lipid A from *S. minnesota* R595. Other fatty acid substitution patterns are encompassed by this disclosure; the essential feature is that the material be de-3-O-acylated.

Thus, one embodiment of this invention is directed to the composition of MPL, DPL and LPS in which the position 3 of the reducing end glucosamine is de-O-acylated. These compounds as indicated above are referred to as d3-MPL, d3-DPL, and d3-LPS, respectively.

Also as indicated above, the modified lipopolysaccharides of the present invention are prepared by subjecting the compounds to alkaline hydrolysis under conditions that result in the loss of but a single fatty acid from position 3 of the lipid A backbone.

The β-hydroxymyristic at position 3 is unusually labile in alkaline media. It requires only very mild alkaline treatment to completely de-3-O-acylate lipid A and LPS. The other ester linkages in lipid A and LPS require somewhat stronger conditions before hydrolysis will occur, so that it is possible to selectively deacylate these materials at position 3 without significantly affecting the rest of the molecule. The reason for the unusual sensitivity to alkaline media of the ester-linked β-hydroxymyristic at position 3 is not known at this time.

Although alkaline hydrolysis procedures are known, it is important to choose conditions that do not cause further hydrolysis beyond the ester linkage to the β-hydroxymyristic at position 3.

In general, the hydrolysis can be carried out in aqueous or organic media. In the latter case, solvents include methanol (alcohols), dimethyl sulfoxide (DMSO), dimethylformanide (DMF), chloroform, dichloromethane, and the like as well as mixtures thereof. Combinations of water and one or more of these organic solvents also can be employed.

The alkaline base can be chosen from among various hydroxides, carbonates, phosphates and amines. Illustrative bases include the inorganic bases such as sodium hydroxide, potassium hydroxide, sodium carbonate, potassium carbonate, sodium bicarbonate, potassium bicarbonate, and the like, and organic bases such alkyl amines and include, but are not limited to, diethylamine, triethylamine and the like.

In aqueous media, the pH is typically between approximately 10 and 14 with a pH of about 12 to about 13.5 being the preferred range. The hydrolysis reaction is typically carried out at a temperature of from about 20° to about 80° C., preferably about 50° to about 60° C. for a period of about 10 to about 30 min. For example, the hydrolysis can be conducted in 3% triethylamine in water at room temperature (22°-25° C.) for a period of 48 hrs. The only requirement in the choice of temperature and time of hydrolysis is that de-O-acylation occurs to remove only the β-hydroxymyristic at position 3.

In practice, it has been found that a particularly desirable hydrolysis method involves dissolving liquid A or monophosphoryl lipid A in chloroform:methanol 2:1 (v/v), saturating this solution with an aqueous buffer consisting of 0.5M Na_2CO_3 at pH 10.5, and then to flash evaporate the solvent at 45°-50° C. under a vacuum for an aspirator (approximately 100 mm Hg). The resulting material is selectively deacylated at position 3. This process can also be carried out with any of the inorganic bases listed above. The addition of a phase transfer catalyst, such as tetrabutyl ammonium bromide, to the organic solution prior to saturating with the aqueous buffer may be desirable in some cases.

In preparing the modified lipopolysaccharides of this invention, it is deemed highly important that LPS can be deacylated at position 3 without causing any changes in the O-antigen or core regions or in the structure of the lipid A component except for loss of the labile fatty acyl residue. There are several implications of this result with respect to possible uses of the de-3-O-acylated compounds. For example, vaccines against gram negative bacteria and/or endotoxin can be generated using LPS that has been treated in the manner of this disclosure which results in a preparation with low endotoxicity but with the same antigenic attributes as the parent material, and which is able to act as its own adjuvant. Such preparations may be able to promote a strong specific immune response without the toxic effects generally associated with LPS-based vaccines.

Another implication is that lipid A that has been detoxified by de-3-O-acylation, since it still contains both phosphates, may have greater immunostimulatory activities than lipid A that has been detoxified by the prior art method involving acid hydrolysis to remove the reducing end phosphate. For example, acid hydrolyzed LPS, which is referred to as monophosphoryl lipid A (MPL), is less mitogenic with respect to B-lymphocyte proliferation than is d3-LPS. Thus, de-3-O-acylated lipid A and LPS may be more potent immunostimulators than is MPL. Furthermore, because of structural differences between MPL and de-3-O-acylated lipid A and LPS, the latter compounds may exhibit a different spectrum of beneficial biological activities than does MPL.

It is therefore viewed as a significant advance to be able to reduce the endotoxicity of LPS without eliminating its antigenic attributes or its immunostimulating activity. LPS subjected to mild alkaline hydrolysis can be used to immunize warm blooded animals including humans, thus conferring protection against gram-negative septicemia and associated endotoxemia.

A further advantage of d3-LPS and d3-DPL relative to MPL is that these materials, since they possess both of the phosphate groups present in lipid A, may exhibit enhanced activities relative to MPL, which is lacking the phosphate moiety at the 1 position. This has already been found with respect to mitogenicity; d3-LPS is as mitogenic as the parent LPS, whereas MPL is only about half as mitogenic.

Finally, the conditions used to effect the mild alkaline hydrolysis disclosed herein are, in some cases, easier to attain than those of the prior art methods for detoxifying LPS or lipid A. For example, as mentioned above, lipid A can be detoxified by dissolving it in a solution chloroform:methanol 2:1 (v/v), saturating this solution with an aqueous buffer consisting of 0.5M Na_2CO_3 at pH 10.5, and then evaporating the solvent at 45°-50° C. This method is also effective in removing the residual endotoxicity which is usually found in crude preparations of MPL, and which is typically removed by chromatographic purification. Thus, milk alkaline hydrolysis can obviate the need for the costly and time-consuming chromatography steps which are generally required in order to fully-detoxify preparations of MPL.

The lipopolysaccharide which is free of the β-hydroxymyristic acid residue, can be covered from the reaction medium in relatively pure form.

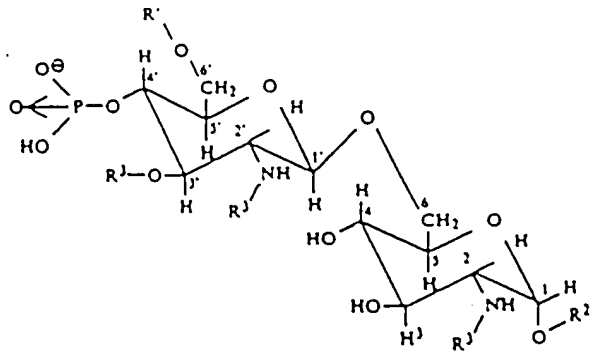
Although the present invention is particularly useful for de-3-O-acylating MPL, it is applicable to lipopolysaccharides in general. Lipopolysaccharides represent a biologically active class of substances and chemically are made up of a polysaccharide portion, the O-specific chains (O-antigen) and the core, and a covalently bound lipid, lipid A. Lipid A represents the endotoxically active region of lipopolysaccharides, while the polar

polysaccharide part serves as a solubilizing carrier. Lipid A of Salmonella consists of a backbone of β-1,6-linked D-glucosamine disaccharide units which are substituted at positions 1 and 4' by phosphate-residues and at position 6' by the core polysaccharide. The other hydroxyl and the amino groups of the backbone are acylated by long-chain fatty acids, of which lauric, myristic, palmitic and 3-hydroxymyristic acid predominate.

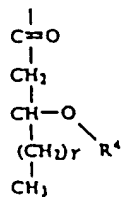
The term "monophosphoryl lipid A", "(MPL)" or "(MLA)" as used herein is meant to designate the monophosphoryl lipid A of structure I and is obtained from lipopolysaccharides such as *Salmonella minnesota* R 595, *Escherichia coli*, and the like. MLA is reported by N. Qureshi et al. Journal of Biological Chemistry, Vol 260, No. 9, pages 5271-5278 (1985).

Accordingly, the lipopolysaccharide compounds of this invention after de-3-O-acylation, can also be represented by the formula.

A lipopolysaccharide compound of the formula



wherein R^1 is selected from the group consisting of hydrogen and the core component of enterobacterial lipopolysaccharide, with or without the O-antigen present, R^2 is selected from the groups H and PO_3H_2, and a R^3 is selected from the group consisting of H, β-hydroxymyristoyl, and a 3-acyloxyacyl residue having the formula:



wherein R^4 = $-C-(CH_2)_Y-CH_3$.

and wherein X and Y can have a value of from 0 up to about 20 and preferably 10, 12 or 14.

The compounds prepared by the present invention are therefore substantially pure compounds and not mixtures of partially de-O-acylated compounds.

All of the uses that are disclosed in the literature for MPL can be entertained with respect to d3-MPL and, especially, d3-DPL. These include (a) use as an adjuvant, (b) protection against radiation, (c) protection against gram negative septicemia and associated endotoxemia, (d) protection against non-specific infectious challenges, and (e) treatment of neoplastic disease. The de-3-O-acylated materials are used in the same way as MPL in all of these applications, i.e., at the same doses, in the same combinations.

Additionally, d3-LPS can be used as a vaccine against gram-negative infections. In this regard, the material is prepared from LPS obtained from either a wild-type strain of a gram negative organism or else from a strain that has a partially-complete (and therefore antigenically cross-reactive) core region (e.g. *E. coli* J5). Such d3-LPS can be administered either in saline, in a lipid emulsion system, or in an oil-in-water emulsion (1-2% squalane or squalene, 0.2% Tween 80). In the latter case, other bacterially-derived immunostimulants (CWS, TDM) can be used in combination with D3-LPS. The amount of d3-LPS per dose is between about 10 and about 1000 μg, and preferably between about 20 and about 200 μg. CWS and TDM, if used, and at similar levels per dose.

The following examples are illustrative of the present invention.

EXAMPLE 1

Removal of the β-Hydroxymyristic Acid at Position 3 of *Escherichia coli* D31M4 MPL by Treatment with Organic Alkaline Media

580 mg of crude *E. coli* D31m4 MPL was dissolved in 250 mls of chloroform:methanol 2:1 (v/v). This solution was transferred to a 1 liter separatory funnel, where it was washed with 100 mls 0.5M Na_2CO_3, pH 10.5. The organic phase was removed, and the solvent was stripped off by flash evaporation using a water aspirator and a bath temperature of 45° C. The resulting residue contained 615.9 mg of de-3-O-acylated MPL (d3-MPL), as judged by thin layer chromatography (Silica gel 60, chloroform:methanol:water:ammonium hydroxide 50:31:6:2 (v/v); plates visualized by spraying with ammonium molybdate in ethanol (10% w/v) and charring).

EXAMPLE 2

The 50% Lethal Dose in Chick Embryos ($CELD_{50}$ of Crude MPL Before and After De-3-O-Acylation

MPL and d3-MPL (prepared in Example 1, above) were dispersed in sterile water containing 0.2% triethylamine (TEA; v/v) to a concentration of 2.0 mg/ml. An ultrasonic bath and mild warming (45°-50° C.) promoted solubilization. To these solutions were added equal volumes of 1.8% NaCl (w/v), giving final solutions that contained 1.0 ug/ml MPL or d3-MPL, 0.9% NaCl (w/v), and 0.1% TEA (v/v). The toxicity of these solutions in 11 day-old chick embryos was then assessed by the method of Milner and Finklestein (J. Infect. Diseases 116, 259: 1966). The chick embryo 50% lethal doses ($CELD_{50}$) were calculated by the method of Reed and Muench (Am. J. Hyg. 27, 493: 1938). The $CELD_{50}$ of the crude MPL used in Example 1 was found to be less than 1 μg. On the other hand, the d3-MPL did not kill any chick embryos even at 20 μg, the highest dilution tested.

EXAMPLE 3

De-3-O-Acylation of *Salmonella minnesota* R595 LPS by Treatment with Aqueous TEA

Into a 4 ml screw-top vial was placed 10.1 mg *S. minnesota* R595 LPS. 2.0 ml sterile water was added to the vial, which was then capped and sonicated for 3

min. at room temperature. The vial was then placed in a boiling water bath. After 5 min. the vial was removed from the bath and 67 ul TEA was added to the solution, with stirring. The vial was capped and allowed to stand at room temperature for 43 hr. At this time, the extent of de-3-O-acylation was assessed by first subjecting a small portion of the reaction solution to acid hydrolysis, in order to convert all of the LPS to MPL. This was accomplished by adding 0.3 ml of 0.47N HCl to a 0.2 ml aliquot of the reaction solution, then placing the acidified solution into an oil bath (130° C.) for 10 min. The solution was stirred during this time. The solution was then cooled in an ice-water bath, and the MPL was extracted using 1.0 ml of chloroform:methanol 2:1 (CM 2:1; v/v). A control solution was prepared by dispersing 1.00 mg LPS in 0.2 ml water plus 6.7 ul TEA, adding 0.3 ml of 0.47N HCl, incubating in a 130° C. oil bath for 10 min., cooling and extracting with CM 2:1. The alkaline-hydrolyzed material and the control were then analyzed by TLC, as described in Example 1. TLC revealed that almost all of the MPL from the TEA-treated LPS was de-3-O-acylated, which indicated that the TEA treatment had resulted in the production of d3-LPS. The control sample, which had not been exposed TEA for the extended period, appeared identical to MPL from untreated LPS. The TEA hydrolysis reaction was therefore judged to be complete. The remaining reaction mixture was dialyzed against distilled water (6,000–8,000 MWt cutoff) and lyophilized, yielding 8.45 mg d3-LPS.

EXAMPLE 4

Biological Activity of d3-LPS

The endotoxicity of the d3-LPS prepared in example 3 was compared with that of the starting LPS using the $CELD_{50}$ assay, as described in Example 2. The activities of LPS and d3-LPS were also evaluated in a lymphocyte proliferation assay, based on uptake of ^3H-thymidine by murine spleen cells following exposure to these materials. The results from these assays are shown in Table 1. They indicate that d3-LPS, while much less endotoxic than the parent LPS, is still a potent mitogen.

TABLE 1

The effect of de-3-O-acylation on the biological activities of *S. minnesota* R595 LPS.

Sample	$CELD_{50}$[a]	Mitogenicity[b] C3H/FeJ[c]	Mitogenicity[b] C3H/HeJ[c]
LPS	0.03 μg	33.9	4.4
d3-LPS	1.4 μg	30.0	5.2

Notes:
[a]The dose necessary to cause 50% mortality in 11 day old chick embryos.
[b]Lymphocyte proliferation assay, based on uptake of ^3H-thymidine by murine spleen cells. The numbers represent the ratio of ^3H counts in stimulated cells to counts in unstimulated cells.
[c]C3H/FeJ mice are LPS-responsive; C3H/HeJ mice are LPS-unresponsive.

EXAMPLE 5

The Rate of De-3-O-Acylation and Detoxification of *S. minnesota* R595 Diphosphoryl Lipid A (DPL) in Organic Alkaline Media

Into each of 4 100×16 mm test tubes was placed 2.0 mg *S. minnesota* R595 DPL. To each tube was added 5 ml CM 2:1 and 2 ml 0.5M Na_2CO_3 pH 10.5. The test tubes were vortexed, centrifuged for 5 min at 3000 g, and the organic layers were withdrawn and transferred to clean test tubes. These solutions were then incubated for varying periods of time at 51°–52° C. (0, 2, 5, and 10 min). The reactions were quenched at the indicated times by placing the tubes in an ice-water bath and adding ice chips to the solutions. After about 30 sec. 2.0 ml of 0.1N HCl was added to each test tube, and the tubes were vortexed and centrifuged. The organic layers were transferred to clean test tubes and washed with distilled water (plus ice chips). Finally, the organic layers were evaporated under a stream of nitrogen. A 0.4 mg portion of each residue was subjected to acid hydrolysis by the method described in Example 3, in order to convert the residues to the corresponding MPLs. The MPLs were then analyzed by TLC as described in Example 1, and the endotoxicities of the corresponding DPL residues from each time point were measured with the $CELD_{50}$ assay, as described in Example 2. The results are summarized in Table II.

TABLE II

The Rate of De-3-O-Acylation and Detoxification of *S. minnesota* R595 Diphosphoryl Lipid A (DPL) in Organic Alkaline Media.

Incubation Time[a]	Extent of De-3-O-Acylation[b]	$CELD_{50}$[c]
0 min	None	0.085 μg
2	Half	NT
5	Almost complete	1.78
10	Complete	10 μg

Notes:
[a]The time each tube was incubated at 51–52° C.
[b]As judged visually from the TLC appearance of the MPL corresponding to the DPL at each time point.
[c]The dose necessary to cause 50% mortality in 11 day-old chick embryos.

Although the invention has been illustrated by the preceding examples, it is not to be construed as being limited to the materials employed therein but rather, the invention relates to the generic area as herein before disclosed. Various modifications and embodiments thereof can be made without departing from the spirit or scope thereof.

What is claimed is:

1. A method for modifying a lipopolysaccharide to selectively remove only the β-hydroxymyristic acyl residue that is ester-linked to the reducing-end glucosamine at position 3 of said lipopolysaccharide, which comprises subjecting said lipopolysaccharide to alkaline hydrolysis sufficient only to remove β-hydroxymyristic acid from position 3 without removal of other fatty acids from the lipopolysaccharide molecule and recovering said lipopolysaccharide free of said residue.

2. The method of claim 1 wherein said lipopolysaccharide is enterobacterial lipopolysaccharide.

3. The method of claim 1 wherein said lipopolysaccharide is monophosphoryl lipid A.

4. The method of claim 1 wherein said lipopolysaccharide is diphosphoryl lipid A.

5. The method of claim 1 wherein said hydrolysis is conducted in the presence of sodium carbonate.

6. The method of claim 1 wherein said hydrolysis is conducted in the presence of triethylamine.

7. The method of claim 1 wherein said hydrolysis is conducted in an organic medium.

8. The method of claim 1 wherein said hydrolysis is conducted in an aqueous medium.

9. The method of claim 1 wherein said hydrolysis is conducted at a pH of from about 10 to about 14 and at a temperature of from about 20° to about 80° C.

10. A method for removing from lipid A or monophosphoryl lipid A, only the β-hydroxymyristic acyl

residue that is ester-linked to the reducing-end glucosamine at position 3 of said lipid A, which method comprises the steps of:

(a) dissolving said lipid A in an inert organic solvent;

(b) saturating said solvent with an aqueous buffer comprised of an alkaline compound, at a pH of from about 10 to about 13;

(c) flash evaporating said solvent under a vacuum at a temperature of between about 40° C. and about 60° C.; and

(d) recovering said lipid A.

11. The method of claim 10 wherein said lipid A is monophosphoryl lipid A.

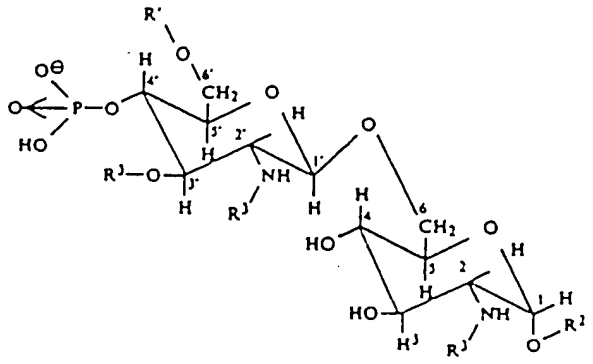
12. The method of claim 10 wherein said inert, organic solvent is a mixture of chloroform and methanol.

13. The method of claim 10 which is effected in the presence of a phase transfer catalyst.

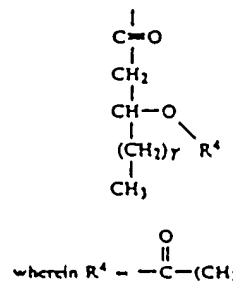
14. The method of claim 13 wherein said phase transfer catalyst is tetrabutyl ammonium bromide.

15. A lipopolysaccharide from which the β-hydroxymyristic acyl group has been removed from position 3.

16. An essentially pure lipopolysaccharide compound of the formula:



wherein R^1 is selected from the group consisting of hydrogen and the core component of enterobacterial lipopolysaccharide, with the O-antigen present, R^2 is selected from the groups H and PO_3H_2, and R^3 is selected from the group consisting of H, β-hydroxymyristoyl, and a 3-acyloxyacyl residue having the formula:



wherein $R^4 = -C(=O)-(CH_2)_x-CH_3$.

and wherein X and Y have a value of from 0 up to about 20.

17. The compound of claim 16 wherein R^1 and R^2 are hydrogen.

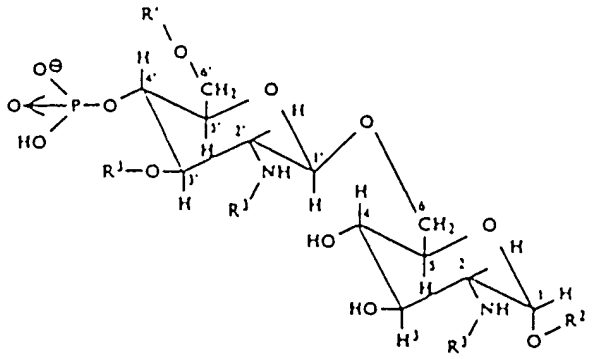
18. The compound of claim 16 wherein R^1 is hydrogen and R^2 is PO_3H_2.

19. The compound of claim 16 wherein R^1 is the core component of enterobacterial lipopolysaccharide and R^2 is PO_3H_2.

20. The compound of claim 16 wherein R^1 and R^2 are hydrogen, and R^3 is the residue of Formula III.

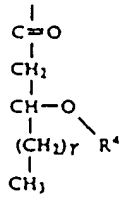
21. The compound of claim 20 wherein X has a value of 10, 12 or 14 and Y is 10.

22. An essentially pure lipopolysaccharide compound of the formula:



wherein R^1 is selected from the group consisting of hydrogen and the core component of enterobacterial lipopolysaccharide, without the O-antigen present, R^2 is selected from the groups H and PO_3H_2, and R^3 is selected from the group consisting of H, β-hydroxymyristoyl, and a 3-acyloxyacyl residue having the formula:

(III)



wherein $R^4 = -C(=O)-(CH_2)_x-CH_3$.

and wherein X and Y have a value of from 0 up to about 20.

23. A pharmaceutical composition comprising a substantially pure, modified lipopolysaccharide, having no β-hydroxymyristic acyl residue that is ester-linked to the reducing-end glucosamine at position 3, and a pharmaceutically acceptable carrier.

24. The pharmaceutical composition of claim 23 wherein said lipopolysaccharide is enterobacterial lipopolysaccharide.

25. The pharmaceutical composition of claim 23 wherein said lipopolysaccharide is monophosphoryl lipid A.

26. The pharmaceutical composition of claim 23 wherein said lipopolysaccharide is diphosphoryl lipid A.

* * * * *

EXHIBIT 13

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re: **Prieels et al** Date: 19 November 1996

Serial No.: 08/356,372 Group Art Unit: 1813

Filed: 17 February 1995 Examiner: L Smith

DECLARATION OF: DR NATHALIE GARCON, UNDER 37 CFR 1.132

To the Commissioner of Patents and Trademarks:

I, Dr Nathalie Garcon DECLARE that:

1. I have received the following academic qualifications:

   Pharm.D from Caen University (France) 1993
   PhD from Lyon University (France) 1996

   I am responsible for vaccine formulation in the applicant Company and I am also a co-inventor of the above identified patent application.

2. A number of experiments have been conducted with a vaccine formulation containing the immunostimulants 3 D- MPL and QS21. I shall deal with four examples in turn: First and second experiments concerned with two different hepatitis B Surface antigen formulations, third a formulation in relation to HIV antigen gp120, and last in relation to a formulation based on an RSV vaccine comprising the hybrid antigen FG. The results below as well as the results shown in the patent application, demonstrate the potent nature of the adjuvant formulation and its broad applicability.

3. **Antibody response to Hepatitis B S antigen formulated with alum, 3D-MPL (MPL) or QS21**

   Formulations were as described in the application. Balb/c mice were inoculated with 70ul of test formulation twice 14 days apart. Sera was collected 14 days after the second inoculation and assayed for total specific Ig G antibodies. The results are depicted in figure 1 attached and show that when 25ug 3 D MPL is added to QS21 formulations a clear synergistic increase in antibody titres is seen when compared with either QS21 or 3 D-MPL alone.

4. **Hepatitis B SL***

In a similar experiment described above mice were immunised with various formulations of adjuvants. The antigen is a mixed particle comprising the S antigen of Hepatitis B and a modified L protein. The combined formulation of the invention significantly enhanced the production of IgG2a antibodies (see Table 1).

5. **HIV gp120**

Mice were immunised I.M with 20μg of HIV gp120 formulated with various adjuvant formulations. The result show (Table 2) that the formulation of the invention synergistically enhances Interferon γ responses and Interlenkin IL2 production.

6. **RSV FG**

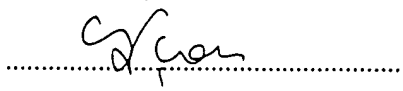
Mice were immunised once with 1μg FG in various adjuvant formulation and total IgG measured. The results are depicted in Table 3. Superior performance in terms of antibody produced was achieved by the vaccine of the invention.

In separate experiments, mice were inoculated with 1μg FG and antibody and cell mediated responses were measured. The results show (Table 4) that the vaccine of the present invention provide superior results to the other formulations tested.

7. **Conclusions**

In conclusion, the vaccine formulated of the invention is highly potent and is of broad applicability.

8. All statements made herein are of my own knowledge and are true, and that all statements made on information and belief are believed to be true, and further that the statements are made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001, Title 18, of the United States Code and that such wilful statements may jeopardise the validity of the above-identified patent application or any patent issued thereon.



NATHALIE GARCON **Dated: 19 November 1996**

Table 1 - Immune response to Hepatitis B antigen, SL* in various adjuvant formulations

	IgG	IgG2a	IFN-g(pg/ml)	IL5 (pg/ml)
3D-MPL/QS21	27326	23038	2299	<31
alum	4215	122	14543	1155
alum/3D-MPL	25498	13823	3223	542

Table 2 - HIV gp120 - Immune response - in various adjuvant formulations

Adjuvant	Total IgG	IgG1	IgG2a	IgG2b	IgG3	Proliferation (CPM)	Il2 (IU/ml)	Il5 (pg/ml)	IFN-g (ng/ml)
PBS	3.3	2.4	1.5	2.5	1.5	25000	0.15	350	2.5
3D-MPL	4.8	3.5	3.2	4	3	25000	0.2	100	5
QS21	3.5	3.2	2.8	3.5	2.5	50000	0.4	250	15
3D-MPL/QS21	4.8	3.5	3	3.8	3	30000	0.8	250	27

Table 3 - RSV FG formulations - IgG response

	log IgG
3D-MPL	10
QS	8.5
3D-MPL/QS	11
no antigen	8.3

Table 4 - Experiment 2 - RSV FG formulations

	IgG1	IgG2a	IgG2b	stimulation index (CMI)	
				spleen	lymph node
3D-MPL	61	24	15	3	15
3D-MPL/QS21	34	52	14	35	94

Cba9304: Measure of IgG against HBSag (14d post2)